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# **ABIOTIC AND BIOTIC STRESS IN PLANTS - RECENT ADVANCES AND FUTURE PERSPECTIVES**

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Edited by **Arun K. Shanker**  
and **Chitra Shanker**

## **Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives**

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## Preface

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The impact of global climate change on crop production has emerged as a major research priority during the past decade. Several forecasts for coming decades project increase in atmospheric CO<sub>2</sub> and temperature and changes in precipitation, resulting in more frequent droughts and floods. Therefore, programmes to develop climate ready varieties with abiotic stress tolerance traits such as temperature and drought tolerance and biotic stress tolerance traits such as insect pest and pathogen resistance in combination with high yield in various important crops should be initiated urgently. Successful adaptation to climate change requires long-term investments in strategic research and new policy initiatives that mainstream climate change adaptation into development planning. A very crucial and highly productive role is envisaged here for basic sciences in identifying metabolic alterations, stress signalling pathways, metabolites and the genes controlling tolerance responses to stresses and in engineering and breeding more efficient and better adapted new crop cultivars. Plant traits that favour yield and also have a direct effect on the mechanism of tolerance is one of the important characteristics that has to be considered when developing climate ready crops. Plant engineering strategies for abiotic stress tolerance have been focused largely on the expression of genes that are involved in osmolyte production; genes encoding ROS scavenging enzymes, LEA proteins, and heterologous enzymes with different temperature optima; and genes for molecular chaperons and transcription factors and proteins involved in ion homeostasis and engineering of cell membranes. The practical value of any genes or pathways for stress tolerance in crop plants can only be useful if there is evidence of superior performance in the field, especially in terms of yield. Selection for yield and stress tolerance *per se* necessitates a “top-down” approach, starting from the dissection of the complex traits to components. Marker-assisted selection (MAS) for abiotic stress-related traits should preferably target ‘major’ QTLs characterized by a sizeable effect, consistent across germplasm and with a limited interaction with the environment. To achieve such a goal, an interdisciplinary and interinstitutional approach would be needed with well-defined targets on crops and problems prioritized at the national level.

Climate change and agriculture are interconnected processes, both of which take place on a global scale. Agriculture is particularly vulnerable to climate change. Higher temperatures will tend to reduce yields of many crops; it may encourage weed and pest proliferation. Changes in precipitation patterns increase the probability of short-run crop failures and long-run production falloffs. Although there are increases in the yield of some crops in some regions of the world, the overall impacts of climate change on agriculture are expected to be negative, threatening global food security. In developing countries, climate change will cause decline in the yield of the most important crops; South Asia will be particularly hard hit. Climate change will have varying effects on irrigated yields across regions, but irrigated

yields for all crops in South Asia will experience a large decline. Climate change will result in additional price increases for the most important agricultural crops – rice, wheat, maize, and soybeans. Higher feed prices will result in higher meat prices; as a result, climate change will reduce the growth in meat. It is important to assess the effects of global climate changes on agriculture; this will help to properly anticipate and adapt farming to maximize agricultural production in a sustainable way. The consequence of climate on agriculture is related to variabilities in local climates rather than in global climate patterns. The Earth's average surface temperature has increased by 0.83°C since 1880. Therefore, it is important for any assessment to be done individually, considering each local area. It is important to note that not all effects of climate change are negative; favourable effects on yield can be seen by realization of the potentially beneficial effects of carbon dioxide on crop growth and increase of efficiency in water use.

Agricultural productivity is sensitive to two broad classes of climate-induced effects: the direct effects because of changes in temperature, precipitation and carbon dioxide concentrations and the indirect effects through changes in soil moisture and the distribution and frequency of infestation by pests and diseases. The main direct effect is generally seen on the duration of the crop growth cycle. Duration of crop growth cycles is related to temperature. Therefore, an increase in temperature will speed up development. In the case of an annual crop, the duration between sowing and harvesting will shorten; for example, the duration in order to harvest a maize crop could shorten between 1 and 4 weeks. The shortening of such a cycle could have an adverse effect on productivity because senescence would occur sooner. In India, an impact of 1–2°C increase in mean air temperature is expected to decrease rice yield by about 0.75 t ha<sup>-1</sup> in efficient zones and 0.06 t ha<sup>-1</sup> in coastal regions and the impact of 0.5°C increase in winter temperature is projected to reduce wheat yields by 0.45 t ha<sup>-1</sup>. Furthermore, crops may experience both low and high weather extremes such as drought and flood and heat and chilling in a single cropping season, and such changes will have varying and complex impacts on agricultural production. Reductions in yields as a result of climate change are predicted to be more pronounced for rain-fed crops than in irrigated crop and under limited water supply situations because there are no coping mechanisms for rainfall variability. Crop growth and yield can be impaired in diverse ways by either high day or high night temperatures and, in addition, soil temperatures also play an important role in the response of crops to heat stress. Additional challenge to temperature increase stems from the fact that higher temperatures will increase the rate at which plants lose moisture, resulting in increased transpiration and water loss. Temperature affects the stages of development of crops during its progress to physiological maturity; the main stages in development of food grain crops that are sensitive to temperature are (i) germination, (ii) canopy and leaf area development, (iii) flowering and reproductive development and (iv) grain development – anthesis to maturity. Further, climate change will indirectly affect crop productivity by changing pest and disease dynamics. Direct effects of pathogens or other organisms can be the induction of resistance or susceptibility and its associated cost or benefit to the host plant. The likelihood of most damaging impacts of diseases and pest can be expected especially in cereals such as wheat and rice. These are likely to have a large impact in terms of food security under climate change scenarios as seen in the case of wheat. Changes in the levels of CO<sub>2</sub>, ozone and UV-B will have an influence on diseases by modifying host physiology and resistance mechanisms. Furthermore, changes in temperature, precipitation and the frequency of extreme events will influence disease epidemiology. An acute change that may arise in the host as an outcome of climate change and the subsequent indirect ef-

fects on the pathogen is a possible outcome. Changes in geographical distribution will possibly alter the comparative importance and range of diseases and may give rise to new disease complexes. Evolution of pathogen populations may hasten from enhanced UV-B radiation and increased fecundity under elevated CO<sub>2</sub>. Consequently, host resistances may be overwhelmed more swiftly; specifically, increases in leaf waxes and epidermal thickness as a result of increased CO<sub>2</sub> atmospheres can result in the host exhibiting higher physical resistance to some pathogens. Carbon dioxide-induced alterations in the architecture of a crop could lead to increased humidity inside the canopy and can create additional favourable condition for pathogen survival. In addition, high speed winds and cyclones can contribute to increased dispersal of airborne plant pathogens such as rusts, splash-borne pathogens such as bacteria and windborne insects and vectors such as aphids and psyllids.

Soil and soil water will be adversely affected by climate change, and this in turn will lead to reduction in the yield of many crops. First-generation climate-carbon cycle models suggest that climate change will suppress carbon accumulation in soils and could even lead to a net loss of global soil carbon over the next century. Changes in soil carbon status are also a matter of concern under changing temperature and changing rainfall regimes; soil carbon is not only important for growth and development of the crop but also for retention of water and nutrients and as an energy source for decomposition process in the soil. The risk of increased erosion is imminent in soils of dry agro ecosystems. High and extreme precipitation will increase runoff primarily due to the inability of the soils to absorb and hold water. Extended dry periods will reduce vegetation cover, which again will result in substantial runoff. Such erosion events occurring frequently will lead to ecosystem change and also loss of soil nutrients. In addition, aridity can hinder surface decomposition and nutrient recycling, thereby affecting crop productivity.

A comprehensive understanding of biotic and abiotic stress, especially the mechanism and tolerance aspects and strategies for adaptation, across the full range of warming scenarios and regions would go a long way in preparing for climate change. A multipronged strategy of using indigenous coping mechanisms, wider adoption of the existing technologies and concerted research and development efforts for evolving new technologies are needed countering biotic and abiotic stress. This multi-authored edited compilation attempts to put forth an all-inclusive picture wherein most aspects of stress will be dealt with. The main purpose of the book, therefore, is to synthesize and present information for developing strategies to combat plant stress. Our effort here is to present a judicious mixture of basic as well as applied research outlooks so as to interest workers in all areas of plant science. We trust that the information covered in this book would bridge the much researched area of stress in plants with the much needed information for evolving climate ready crop cultivars to ensure food security in the future.

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# Genetic Basis of Abiotic and Biotic Stress Tolerance

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# **Transcriptional Network Involved in Drought Response and Adaptation in Cereals**

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Additional information is available at the end of the chapter

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## **Abstract**

Drought is the major abiotic stress in many wheat environments, decreasing grain yields and farmer's income. Finding ways to improve drought tolerance in wheat is therefore a global effort. Transcription factors (TFs) play important roles in drought tolerance by stimulating plant's protective genome activities in response to heat and water limitation. TFs are specialized proteins which can bind to specific DNA elements in gene promoters and modulate gene expression in response to various external and internal stimuli. Thus TFs is a crucial part of plant signal transduction pathway mediated by signal receptors, phytohormones and other regulatory compounds. The activities of TFs are closely related to their structure, and their binding specificity is determined by the homo-/hetero-dimerization of TFs. The expression of downstream genes may produce a subset of TFs or regulate other functional proteins involved in physiological drought adaptation. Thus, the hierarchic regulations of TF activities, downstream gene expression and protein-protein interaction comprise a complex regulatory network, which participates in drought response and adaptation in cereal crops. Basic mechanisms of this regulatory network have been described, but more insight is needed to find new tools for enhancing cereals' adaptation to drought stress.

**Keywords:** Abiotic stress, cereals, drought, regulatory networks, transcription factors

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## **1. Introduction**

Drought is the major environmental factor that limits crop growth and yield globally. Improving crop performance under water limiting conditions is, therefore, an important research focus of plant scientists around the world. Limited water availability evokes adaptive physiological responses regulated by changes in expression of numerous stress-responsible genes.

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Transcription factors (TFs) are groups of proteins that bind to specific regulatory DNA elements located in gene promoters upstream of transcription initiation sites, repressing or activating target gene expression. Intensive research in recent years has shown that temporal and spatial modulation of stress-related TFs provides an efficient way for plants to deal with unfavourable growth conditions. TFs involved in drought response were identified and characterized in various cereal species, including all major food crops such as in rice [1–4], wheat [5–8], barley [9, 10], and maize [11, 12]. Overexpression of several target genes encoding stress-responsive TFs led to improved survival rate under water limitation in transgenic plants [4, 10, 12–14].

The activities of TFs are closely related to their structure. TFs usually contain a DNA-binding domain (DBD) and a transcriptional activation domain (TAD) [15]. The DBD enables TFs to bind with specific promoter elements of target genes, and TAD mediates regulation of the downstream gene either directly or in cooperation with other proteins. TAD usually represents a low-complexity sequence that prevents protein self-folding and facilitates protein–protein interactions (PPI). The transcriptional response to drought in cereals is controlled by a large number of TFs, which have been grouped into several different families based on their structure and binding specificity. The main TFs discussed in this article belong to the following families: the DRE-binding protein/C-repeat binding factors (DREB/CBF) [1, 13, 16, 17], the NAM/ATAF1/CUC2 (NAC) factors [18, 19], the MYB family [20, 21], the WRKY family [9, 22], the basic leucine zipper family (bZIP) [14, 23–25], and the homeodomain-leucine-zipper (HD-Zip) family [26–28].

When plants suffer water deficiency, receptors from the cell membrane/cell wall sense the extracellular stress signals and convert them into intracellular secondary messengers such as  $\text{Ca}^{2+}$  and inositol phosphate [29]. How exactly the signal is transmitted toward gene activation is still poorly understood and is a subject of intensive multidisciplinary investigations. However, it is well agreed that plant hormones, especially abscisic acid (ABA), play significant role in drought stress-related transcription, in many cases through modulating phosphorylation status of transcription factors and other regulatory proteins.

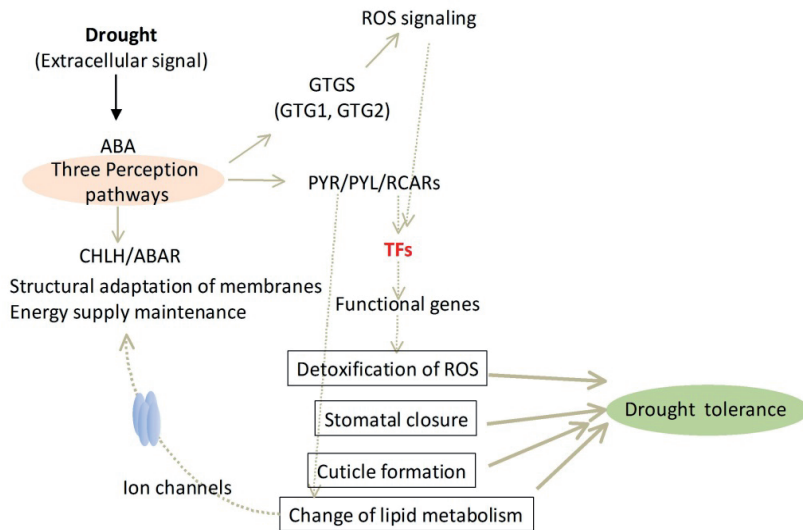
The objective of this article is to review the involvement of TFs in drought response and adaptation in cereals and to illuminate the complexity of the factors and processes involved. The article is subdivided into four sections, which will (1) give examples of drought-related hierarchy in TF interactions regulated by plant hormones, (2) provide an overview of major families of cereal TFs involved in drought response, (3) overview existing data on TF target gene networks activated in response to drought, and (4) describe the homo- and heterodimerization in relation to TF's activities.

## 2. Plant hormone crosstalk in drought relevant regulatory pathways

Phytohormones play critical roles in linking the stress-responsive signaling cascades. ABA is a key plant hormone that functions as a link between environmental stress reception and adaptive transcriptional programs such as the regulation of cellular mechanisms, carbohydrate

and lipid metabolism. Similar to ABA, gibberellic acid (GA) and jasmonic acid (JA) play important functions in cellular stress network signaling. Different receptors have been reported to recognize and bind with these plant growth regulators in order to activate or modulate downstream responses [30].

Drought and ABA-mediated signals are perceived through three main pathways by different receptors (see Figure 1). The first receptor PYR/PYL/RCARs (PYRABACTIN RESISTANCE / PYRABACTIN RESISTANCE-LIKE / REGULATORY COMPONENT OF ABA RECEPTOR-SPYR) binds ABA and inactivates the type 2C protein phosphatases (PP2Cs), which leads to the accumulation of SNF1-RELATED PROTEIN KINASES (SnRK2s) [30]. SnRK2s activate ABA-responsive TFs such as *Arabidopsis* AREB1, AREB2, and AREB3. These TFs regulate ABA-dependent gene expression involved in several physiological processes such as the movement of stomatal guard cells, thereby increasing the tolerance to drought [30]. AtMYB44 is one of the TFs that negatively regulate the target genes coding PP2Cs, which leads to stomatal closure and reduced transpiration losses [31]. Protein-coupled receptor-type G proteins (GTGs) such as GTG1/GTG2 are involved in the second ABA reception pathway, which was first reported in *Arabidopsis* [30]. GTG1/GTG2 proteins are membrane-localized receptors with functions in seedling and pollen tube growth and development, acting through voltage-dependent anion channels. The third ABA receptor is the H subunit of Mg-chelatase (CHLH/ABAR), which regulates the lipid metabolism linked to drought tolerance in plants [30].



**Figure 1.** A schematic model of the signal chain from drought stress perception to physiological responses and drought tolerance.

It was also demonstrated that ABA can increase the transcription level of reactive oxygen species (ROS) network genes [30]. ROS are reactive oxygen-based molecules such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $OH^\cdot$ ), which not only toxically damage cells through membrane peroxidation and de-esterification under environmental stresses but also trigger stress endurance in plants [30]. For example, ABA has been shown to trigger the activity of cytosolic aldehyde oxidase and xanthine dehydrogenase, which separately produce  $H_2O_2$  and  $O_2^-$  in drought [32].

The GA receptor GA INSENSITIVE DWARF1 (GID1) was reported from rice and is a homolog of the *Arabidopsis* GID1a/b/c [30]. GA-responsive TFs GRAS (GA insensitive [GAI], REPRESSOR of *ga1-3* [RGA], and SCARECROW [SCR]) are GA signaling repressors involved in GA-controlled plant development [30]. Subgroup of GRAS, called DELLA proteins, can interact with GID1 and lead to DELLA protein degradation. The downstream gene of DELLA TFs encoding a RING-H2 zinc finger factor XERICO is involved in ABA and GA transduction pathways under abiotic stresses [30]. Further, the DELLA protein RGL3 can be responsive to JA and interact with the JA regulator OsJAZ (jasmonic acid ZIM-domain protein) under drought [30]. Thus, DELLA proteins can be considered as the interface of ABA, GA, and JA signaling pathways in response to water deficiency [30].

The regulation network of TFs plays an important role in stress-relevant hierarchic regulatory pathways. OsNAC10, a NAC TF, can up-regulate the downstream genes encoding AP2 and WRKY TFs involved in ROS detoxification and scavenging for drought response through the ABA synthesis pathway. The mechanisms of plant response to drought include cell wall development and cuticle formation [30]. The promoter region of the gene *OsNAC6* contains various recognition sites such as ABREs, MYBRS, MYCRS, W-boxes, and GCC boxes, which can be separately recognized by TFs AREB/ABF, MYB, MYC, WRKY, and ERF [33]. These TFs are likely to bind to the corresponding *cis*-elements and co-regulate the expression of *OsNAC6* that participate in the ABA induction pathway and abiotic stress response in plants. In the bZIP family, the gene encoding OsbZIP12 was also found to have MYBRS, MYCRS, and W-box motifs in its promoter region, which can be recognized by TFs MYB, MYC, and WRKY, respectively [34]. Besides, OsNAC5 and OsbZip23 might co-regulate the expression of the downstream gene *OsLEA3* since both of them enhance the transcription level of *OsLEA3* [35]. OsDREB1F might interact directly/indirectly with some bZIP family members in the ABA-dependent pathway that activate transcription of the ABA responsive genes *rd29B* and *RAB18* [3]. However, more in-depth studies are needed to identify these events and to explain the underlying mechanism.

### 3. Major families of cereal TFs involved in drought response

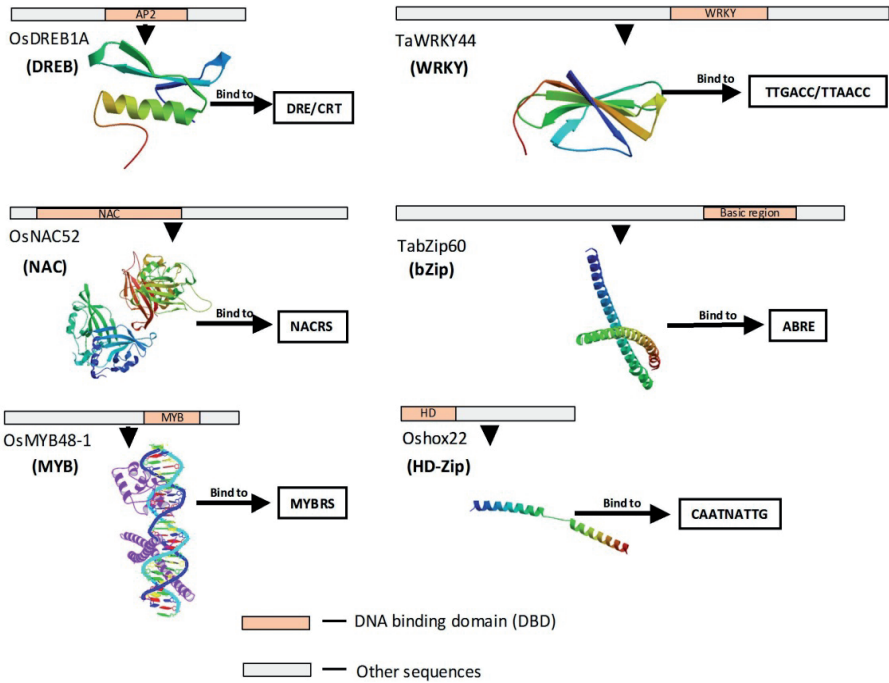
Transcription factors are classified into several family groups mainly based on characteristic amino acid sequences of its conserved DBDs [36, 37]. Of these, the families DREB/CBF, NAC, MYB, WRKY, bZIP, and HD-Zip are the main TFs involved in drought. Their structural features, classification, and representative family members in cereals are summarized in Table 1 and Figure 2.

TF	Species	TF's name	Cis-element recognition	Downstream genes	Accession/locus number	Reference	ABA (D)	Transgenic plants	Stress inducible/tolerance
DREB	Rice	OsDREB1A	DRE/CRT (G/ACCGAC)	e.g., <i>cor15A</i> , <i>rd29A</i> , <i>rd17</i>	AP001168	[1, 129]	D	Rice, <i>Arabidopsis</i>	Drought, salt, cold
		OsDREB1B	DRE/CRT (G/ACCGAC)	U	AY785896	[44]	I	Rice	Drought
		OsDREB1F	DRE/CRT (G/ACCGAC)	<i>rd29A</i> , <i>COR15a</i> , <i>rd29B</i> , <i>RAB18</i>	AY345234	[3]	D/I	Rice, <i>Arabidopsis</i>	Drought, salt, cold
		OsDREB2A	DRE/CRT (G/ACCGAC)	U	XM_485622	[44]	I	Rice	Drought
		OsDREB2B	DRE/CRT (G/ACCGAC)	U	AF300971	[45]	I	Rice	Drought
DREB	Wheat	TaDREB1	DRE/CRT (TACCGACAT)	<i>rd29A</i>	AF303376	[44]	I	Rice, <i>Arabidopsis</i>	Drought, salt, cold
		TaDREB2	DRE/CRT (G/ACCGAC)	e.g., <i>TaRAB16.5</i> , <i>TaWZY2</i> , <i>TaWH10</i>	GU785008	[13]	I	Barley, wheat	Frost, drought
		TaDREB3	DRE/CRT (G/ACCGAC)	e.g., <i>TaRAB16.5</i> , <i>TaWZY2</i> , <i>TaWH10</i>	CU785009	[13]	I	Barley, wheat	Frost, drought
		HvDREB1	DRE/CRT (G/ACCGAC)	<i>rd29A</i>	DQ012941	[47]	D/I	<i>Arabidopsis</i>	Drought, salt, cold
		HvCBF4	DRE/CRT (G/ATCGAC)	e.g., <i>Albice</i> , <i>LRR</i> , <i>CgP450</i>	AF298230	[48]	I	Rice	Drought, salt, cold
Maize	Maize	ZmDREB1A	DRE/CRT (G/ACCGAC)	e.g., <i>COR15A</i> , <i>KNI1</i> , <i>KIN2</i>	AF450481	[11]	I	<i>Arabidopsis</i>	Drought, cold, salt
		ZmDREB2A	DRE/CRT (G/ACCGAC)	<i>rd29A</i> , <i>rd29B</i>	AY108198	[110]	I	<i>Arabidopsis</i>	Drought, heat
		ZmDREB7	DRE/CRT (G/ACCGAC)	U	GRMZM2Q208386	[46]	I	<i>Arabidopsis</i>	Drought
		OSNAC5	NACRS (CAGC)	<i>OsLEA3</i> , <i>Oslog6812100</i>	AK102475	[35, 57, 130]	D	Rice	Drought, salt, cold
		OSNAC6	NACRS (CAGC)	<i>AK104277</i> , <i>AK110725</i>	BD28185.1	[33, 35, 38]	D	Rice	Drought, cold, salt
Rice	Rice	OSNAC9	NACRS	e.g., <i>NCE1</i> , <i>Ga-ATPase</i> , <i>CCR</i>	GS31855	[59]	U	Rice	Drought
		OSNAC10	U	U	U	[60]	D	Rice	Drought, salinity
		OSNAC52	NACRS	e.g., <i>rd29A</i> , <i>rd29B</i> , <i>RD22</i>	AAT44250	[61]	D	<i>Arabidopsis</i>	Drought
		OSNAC05	NACRS	<i>OsLEA3-1</i> , <i>OsPM1</i>	CT829509	[19]	D	Rice	Drought, salt
		SNAC1	U	U	AK067690	[18]	D	Rice	Drought, salt
NAC	Wheat	TaNAC-2	NACRS	e.g., <i>rd29A</i> , <i>rd29B</i> , <i>RD22</i>	U	[52]	D/I	<i>Arabidopsis</i>	Drought
		TaNAC67	NACRS	<i>DREB2A</i> , <i>COR15</i> , <i>AB11</i> , <i>AB12</i>	KF646593	[54]	D	<i>Arabidopsis</i>	Drought, salt, cold
		TaNAC69	NACRS	<i>Chitinase</i> , <i>ZIM</i> , <i>glyoxalase 1</i>	U	[53]	U	Wheat	Drought
		TaSNAC69-1	U	U	AY625682	[131]	U	N	Drought, salinity, heat
		ZmSNAC1	U	U	U	[132]	D	Sorghum	Drought, cold, salt
Maize	Maize	ZmSNAC052	U	U	KM987612	[55]	U	N	Drought, cold
		Zma000584	U	U	KP283536	[55]	U	N	Drought, cold
		Zma006493	U	U	KM670443	[55]	U	N	Drought
		Zma001259	U	U	KM670444	[55]	U	N	Drought, cold, salt
		OsMYB3K-2	MYBRS	<i>Dehydration-responsive element-binding protein 2A</i> , <i>COR15A</i> , <i>RC12A</i>	BAD81765	[20]	U	<i>Arabidopsis</i>	Drought
MYBs	Rice	OsMYB4	U	U	Y11414	[71]	U	Apple	Drought, cold
		TaMYB4E-1	MYBRS	e.g., <i>OsPP2C68</i> , <i>RAB21</i> , <i>OsNCE24</i>	Os01g2410.2	[72]	D	Rice	Drought, salinity
		TaMYB3K1	U	U	HQ236494	[73]	D	N	Drought, salt, cold
		TaMYB30-B	MYBRS	<i>rd29A</i> , <i>ERD1</i>	U	[7]	I	<i>Arabidopsis</i>	Drought
		ZmMYB-R1	U	U	IQ37942	[70]	D	N	Drought, salt, heat, cold
WRKY	Rice	OsWRKY3	U	U	Os03g5980	[81]	D	N	Drought
		OsWRKY4	U	U	Os06g4010	[81]	U	N	Drought, cold, flood
		OsWRKY8	U	U	Os01g02480	[81]	U	N	Drought, cold
		OsWRKY11	U	U	AK106745	[22]	U	Rice	Drought, heat
		OsWRKY18	U	U	Os02g06440	[81]	U	N	Drought, cold, flood
WRKY	Rice	OsWRKY22	U	U	Os01g01080	[81]	U	N	Drought, cold, flood
		OsWRKY24	U	U	Os01g05660	[81, 82]	D	N	Drought, cold

TF	Species	TFs name	Cis-element recognition	Downstream genes	Accession/number	Reference	ABA (Df)	Transgenic plants	Stress inducible/tolerance
WRKY	Rice	OsWRKY42	U	U	Os04g21950	[81]	U	N	Drought, cold
		OsWRKY45	U	U	Os05g14370	[78]	D	<i>Arabidopsis</i>	Drought, disease
		OsWRKY30	U	U	Os02g26430	[81]	U	N	Drought, cold, flood
		OsWRKY53	U	U	Os08g29660	[81]	U	N	Drought, cold, flood
		OsWRKY78	U	U	Os11g29870	[81]	U	N	Drought, cold, flood
	WbKY	OsWRKY84	U	U	Os01g54600	[81]	U	N	Drought, cold, flood
		OsWRKY96	U	U	Os01g14440	[81]	U	N	Drought, cold, flood
		OsWRKY100	U	U	Os09g16510	[81]	U	N	Drought, cold, flood
		TaWRKY2	U	STZ	EU65425	[82]	D	<i>Arabidopsis</i>	Drought, salt
		TaWRKY10	U	U	HQ270327	[83]	D	Tobacco	Drought, salinity
bZIP	Wheat	TaWRKY19	U	<i>Cor6.6, rd28A, rd29B</i>	EU65430	[82]	D	<i>Arabidopsis</i>	Drought, salt, freezing stress
		TaWRKY44	(TTGACG/TTAACC)	e.g., <i>NtGOD, NtAPX, NtCAT</i>	K8827395	[111]	U	Tobacco	Drought, salt, osmotic stresses
	Barley	HvWRKY38	U	U	CA260651	[133]	U	N	Drought
		OsZfp12	ABRE	<i>OsLEA3-1, et al.</i>	AK072062	[23, 92]	D	Rice	Drought, salt
	Rice	OsZfp12	ABRE	<i>LEA3, Rab16</i>	U	[34]	D	Rice	Drought
		OsZfp16	ABRE	<i>LEA3-1, RAB16C</i>	Os02g09830	[91]	D	Rice	Drought
		OsZfp45	ABRE	U	Os05g0569300	[92]	D	Rice	Drought
		OsZfp46	ABRE	e.g., <i>RAB21</i>	AK103188	[98]	D	Rice	Drought, heat, hydrogen peroxide
		OsZfp32, RISRZ5	G-box	<i>OsLEA3, OsTPP1, RAB25</i>	Os06g45140	[24]	I	Rice	Drought, cold
bZIP	Wheat	OsZfp71	ABRE or DRE	<i>OsCAT, OsNHX1, OsMY</i>	Os09g13570	[25]	D	Rice	Drought, salt
		OsZfp72	ABRE	<i>LEA3, Rab16</i>	Os09g28310	[25, 93]	D	Rice	Drought
	Wheat	TaZfp60	ABRE	e.g., <i>AtH29A, AtFD20, AtH29B</i>	K156288, K180655, K1806560	[84]	D	<i>Arabidopsis</i>	Drought, salt, freezing stress
		TaABP1	U	U	HQ166718	[134]	D	Tobacco	Drought, salt, cold
	bZIP	HvZfp13	U	U	U	[86]	D	N	Drought
		HvZfp15	U	U	AK365526.1	[86]	D	N	Drought
		HvZfp18	U	U	AK251589.1	[86]	D	N	Drought
		HvZfp20	U	U	AK359622.1	[86]	D	N	Drought
		HvZfp23	U	U	AK374925.1	[86]	D	N	Drought
bZIP	Barley	HvZfp29	U	U	AK359391.1	[86]	D	N	Drought, cold
		HvZfp34	U	U	AK365092.1	[86]	D	N	Drought
		HvZfp40	U	U	AK249866.1	[86]	D	N	Drought
		HvZfp42	U	U	AK368116.1	[86]	D	N	Drought
		HvZfp49	U	U	AK369418.1	[86]	D	N	Drought
	Maize	HvZfp52	U	U	AK372616.1	[86]	D	N	Drought
		HvZfp53	U	U	AK359129.1	[86]	D	N	Drought
		HvZfp77	U	U	U	[86]	D	N	Drought
		ZmZfp17	U	U	NN_001158672	[89]	D	N	Drought, heat, salt
		ZmZfp37	U	U	GRMZM5C858197	[88]	D	N	Drought
HD-Zip	Maize	ZmZfp72	ABRE	e.g., <i>rd29B, RAB18, HSI1-3</i>	HQ328839	[90]	U	<i>Arabidopsis</i>	Drought, salt, osmotic stress
		ZmZfp74	U	U	GRMZM2G448607	[88]	U	N	Drought
	Rice	ZmZfp112	U	U	GRMZM2G03647	[88]	U	N	Drought
		Osbox22	CAAT (GC) / ATTG	U	AY224440	[27]	D	Rice	Drought, salt
	Maize	OsHv4	U	U	AF145728	[104]	U	Rice	Drought, salt
		Zmhd210	CAATAATTG	U	JN514832	[108]	D	Rice, <i>Arabidopsis</i>	Drought, salt
	U, Unknown; D, ABA-dependent; I, ABA-independent; N, No transgenic								

Table 1. Overview on the main cereal transcription factor family members involved in drought.





**Figure 2.** Schematic representation of domain compositions, secondary structures, and recognition sites of major drought-related TF families. The secondary structures were predicted using SWISS-MODEL (<http://swissmodel.expasy.org/>).

### 3.1. DREB/CBF family

The DREB/CBF family is a member of the AP2/EREBF superfamily of TFs, responsive to several stresses including drought [3, 8]. A cDNA encoding the first identified DREB/CBF family member CBF1 was isolated from *Arabidopsis thaliana* and characterized by Stockinger et al. [38]. DREB/CBF TFs possess about 60 amino acid long AP2 DBD which specifically recognizes a dehydration-responsive C-repeat (DRE/CRT) *cis*-element. The AP2 is a highly conserved domain of DREB family members. It contains two conserved motifs: the YRG and RAYD motifs. The YRG motif is considered to determine DNA binding and the RAYD motif, which forms an  $\alpha$ -helix on the C-terminus, is supposed to play a role in PPI [39]. Drought responsive DREB TFs were also found in other plant species such as *Brassica napus* [40], *Triticum aestivum* [41], *Atriplex hortensis* [17], and *Oryza sativa* [42].

Many reported drought-inducible cereal DREBs were shown to be regulators improving stress endurance. In wheat, the gene *TaDREB1* [41] was induced by drought, salt, and cold. The transgenic barely containing *TaDREB2* and *TaDREB3* [13] showed improved tolerance in drought and low temperature conditions. In rice, 13 transcriptional factors including seven

DREB1 types (OsDREB1A, 1B, 1C, 1D, 1E, 1F, and 1G) and six DREB2 types (OsDREB2A, 2B, 2C, 2D, 2E, and OsAB14) [43] were isolated and analyzed. The overexpression of OsDREB1A [1] and OsDREB1F [3] resulted in transgenic *Arabidopsis* and rice plants with higher tolerance to salt, drought, and low temperature. OsDREB1G, 2A, and 2B were identified to be strong candidates in drought responsive pathways, while OsDREB1E could slightly improve the drought survival rate in transgenic rice [44, 45]. In different wheat cultivars, TaDREB1 was demonstrated to be inducible by drought, salt, low temperature, and ABA [41]. TaDREB2 and TaDREB3 significantly improved frost and drought tolerance in transgenic barley and wheat [13]. In maize, ZmDREB1A [11], -2A [94], and ZmDREB2.7 [46] contributed to drought tolerance. In barley, the gene *HvDREB1* [47] was induced by drought, salt, and low temperature, while the constitutive expression of *HvCBF4* [48] increased the survival rate of transgenic rice under drought.

### 3.2. NAC family

The NAM/ATAF/CUC (NAC) TFs contain a unique feature, a conserved N-terminus DBD and a dissimilar C-terminus regulatory domain, and they are spread across the plant kingdom [37]. The name NAC is an abbreviation of three genes designated as no apical meristem (*NAM*), *Arabidopsis* transcription activation factor (*ATAF*), and cup-shaped cotyledon (*CUC*), which encode proteins containing homologous sequences as the NAC domain [49, 50]. *NAM* isolated from petunia by Souer et al. [49] was the first gene demonstrated to encode a NAC protein, followed by the gene *CUC2* from *Arabidopsis* [50]. Mutation of both genes resulted in the absence of apical shoot meristems [51] and led to floral abnormalities such as the alteration of petal primordia positions during the development stage. These evidences show that the TFs *NAM* and *CUC* play important roles in shoot apical meristem formation and determine the organ primordia positions in the floral meristem [49, 50]. But in relation to abiotic stress, the wheat genes *TaNAC2* [52] and *TaNAC69* [53] were strongly expressed under water deficiency and salinity. *TaNAC67* was found to decrease the cell membrane instability, preventing water loss and enhancing other physiological processes that were considered to be responsive to drought, low temperature, and salt stress [54]. In maize, ZmNAC052, Zma000584, Zma006493, Zma001259z [55], and ZmSNAC1 showed increased transcription levels under water deficiency, indicating their potential role in drought tolerance regulation. In rice, SNAC1 [56], OsNAC5 [57], -6 [58], -9 [59], and -10 [60] altered the root structure for plant adaptation during drought. Further, the overexpression of *OsNAC045* [19] and *OsNAC52* [61] induced ABA sensitivity and conferred drought resistance in transgenic rice and transgenic *Arabidopsis*, respectively.

### 3.3. MYBs family

MYB is a group of ancient TFs found in viruses [62] and eukaryotes such as plants, animals, and fungi [63]. The first gene (c1) identified to encode MYB in plants was from *Zea Mays* [64]. MYB TFs contain a conserved DBD called MYB domain characterized by one to three imperfect repeated amino acid sequences (R1, R2, and R3). Each repeat sequence has around 50–53 amino acids which form three  $\alpha$ -helices [65]. These three  $\alpha$ -helices form a helix-turn-helix structure

when interacting with DNA. The MYB TFs are mostly classified into three classes according to the number of the MYB domain repeats: R1-MYB, R2R3-MYB [66], and R1R2R3-MYB [67]. These TFs participate in responses to dehydration, salt, cold, and drought [20, 68, 69]. In maize, the ZmMYB-R1 was induced by ABA, drought, low temperature, high salt, and heat [70]. The overexpression of rice genes *OsMYB3R-2* [20], *OsMYB4* [71], and *OsMYB48-1* [72] improved the adaptive response to drought and other stresses in transgenic plants. In wheat, TaMYB3R1 [73] and TaMYB30-B [7] were found to be potentially involved in drought adaptation.

### 3.4. WRKY family

WRKY proteins belong to the superfamily WRKY-GCM1 of zinc finger TFs [74]. They exist in numerous plant species [75] and were reported to be involved in several biotic and abiotic stress responses and developmental processes such as embryogenesis and leaf senescence [75]. WRKY family members contain a highly conserved WRKY domain with 60 amino acids comprised of two motifs. One is the conserved WRKYGQK motif on the N-terminus and the other one is a zinc-finger-like motif on the C-terminus [76]. There are three main groups in the WRKY family according to the different number of WRKY domains and the variable structure of the zinc-finger-like motif [76]. Group I has two WRKY domains, whereas groups II and III have one WRKY domain. The zinc finger motifs of the WRKY domain in groups I and II are the same, but different in group III [75]. The group II has been divided into five subgroups by Eulgem et al. [76], designated as IIa, IIb, IIc, IId, and IId, according to the conserved motifs outside the WRKY domain. Within these five subgroups, Zhang et al. [77] distinguished another three new groups (2\_a+2\_b, 2\_c, 2\_d+2\_e). Several drought-related WRKY TFs were found in rice, wheat, and barley [78–80]. In rice, *OsWRKY3*, -4, -8, -18, -22, -24, -42, -50, -53, -78, -84, -96, and -100 were found to be co-expressed in drought and cold stress, and some of them were even expressed in different organs of flooded plants [81]. *OsWRKY11* was identified to be involved in drought and heat response [22]. *OsWRKY45* was found to be sensitive to ABA and considered to play a role in stomatal closure to improve drought and salt tolerance [78]. In barley, *HvWRKY38* was shown to have a function in drought and cold response [9]. In wheat, the overexpression of TaWRKY2, -19 [82], and -10 [83] led to improved drought and salt adaptation in transgenic plants.

### 3.5. bZIP family

The basic leucine zipper (bZIP) family is another big group of TFs involved in diverse functions such as hormone and sugar signaling and organ development [84]. bZIP proteins commonly have a basic region for DNA binding and a conserved leucine zipper motif [85]. These TFs specifically bind to a DNA sequence with a core *cis*-element ACGT-like TACGTA (A-box), GACGTC (C-box), CACGTG (G-box), and an ABA-responsive element (ABRE) [84]. Some bZIP members were identified to participate in transducing ABA-dependent stress signals and were named as ABRE binding proteins (AREBs) or ABRE binding factors (ABFs) [86]. Numerous bZIP proteins were demonstrated or predicted to be involved in abiotic stress response in cereal plants, e.g., 89 in rice [87], 171 in maize [88], and 141 in barley [86]. They were classified into 11 groups A, B, C, D, E, F, G, H, I, S, and U according to the phylogenetic trees and DNA

binding motif [86] in maize, rice, and barley. The overexpression of several cereal bZIP TFs was identified to be in response to drought stress. In barley, 11 HvbZIP members were identified to be down- or up-regulated by drought [86]. In maize, ZmbZIP37, -17, and -112 showed high expression levels in drought stress conditions [88, 89] and the overexpression of ZmbZIP72 enhanced the drought tolerance in transgenic *Arabidopsis* [90]. In rice, OsbZIP12 [34], -16 [91], -23, -45 [92], -71 [25], and -72 [93] play a positive role in drought tolerance through ABA signal, while OsbZIP52 [24] and -46 [94] were suggested to be a negative regulator in water deficiency. In wheat, the gene encoding *TabZIP60* was highly induced by salt, cold, and ABA, and the overexpression of *TabZIP60* enhanced the drought and frost tolerance in transgenic *Arabidopsis* [84].

### 3.6. HD-Zip family

The homeodomain leucine zipper (HD-Zip) family is a group of proteins that are unique to the plant kingdom. All members of the HD-Zip family contain the combination of homeodomain (HD) [6] with a following leucine zipper (Zip or LZ). HD is a conserved protein domain containing a 60 amino acids sequence, which is present in all eukaryotic species [95]. HD is a folded structure with three helices, which are responsible for the specific protein-DNA interactions [95]. HD-Zip family proteins have been classified into HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV [96–100] according to different domain structure and functions. According to Chan et al. [101], HD-Zip I TFs have less conserved motifs than HD-Zip II, and the sequences of HD-Zip I outside the HD-Zip domain are quite different, whereas HD-Zip II TFs have several common sequences outside the HD-Zip domain. The HD-Zip III TFs have four additional amino acids on the conjunction of HD and LZ compared with other three subfamilies [102]. HD-Zip I TFs contain no lipid/sterol-binding domain, designated StAR-related lipid transfer (START) domain, which was found in HD-Zip III and HD-Zip IV TFs [102]. Hence, the special structural feature of HD-Zip I TFs is the presence of HD and leucine zipper and the absence of common sequences outside the HD-Zip domain [103].

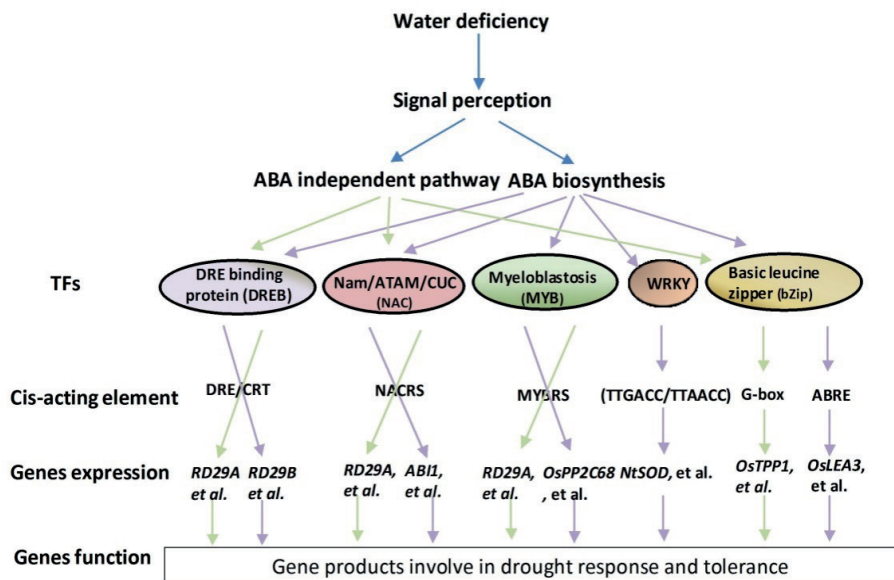
In rice, the *HD-Zip I* subfamily has 14 members: *Oshox4-6*, *Oshox8*, *Oshox12-14*, *Oshox16*, and *Oshox20-25* [104]. Three of them, *OsHOX6*, *OsHOX22* and *OsHOX24*, are homologs of the ABA and abiotic stress-inducible genes *AtHB7* and *AtHB12* in *Arabidopsis* [105]. *OsHOX22* and *OsHOX24* have been identified to be involved in drought, cold, and ABA response. *OsHOX22* is strongly activated by high salinity and ABA, but it is weakly induced by frost [27]. Zhang et al. [27] found that the insertion of T-DNA into the *OsHOX22* promoter region led to a decreased gene expression level of *OsHOX22* and reduced ABA content, but improved drought and salt endurance of rice seedlings. The authors believe that *OsHOX22* is a negative regulator for stress response by regulating an ABA-mediated signal transduction pathway and ABA biosynthesis [27]. The *OsHOX24* promoter has shown strong activation by water deficiency and high salinity [106]. According to the results of Agalou et al. [104], *OsHOX22* and *OsHOX24* can be induced by drought in drought-sensitive and drought-resistant cultivars, whereas *OsHOX6* can only be induced in drought sensitive cultivars. Although the role of *OsHOX6* and *OsHOX24* TFs is still not clear, the homologs of these TFs, *AtHB7* and *AtHB12*, have been found to be involved in ABA modulation by regulating the protein phosphatase 2C

activation and an ABA receptor gene activity [107]. In short, TFs Oshox6, -22, and -24 are responsive to dry conditions, similarly as the *ATHB7* and *ATHB12*, and their involvement in drought response might have relevance for ABA synthesis regulation [104].

In maize, the HD-Zip TF Zmhdz10 was found to play an important role in drought response [108], and in wheat, only two HD-Zip TFs, TaHDZip1-1 and TaHDZip1-2, were reported so far [109]. However, there is no information about the function of wheat HD-Zip proteins in drought response.

#### 4. TFs target gene network activated in response to drought

TFs are involved in target gene network regulation through their DBD interaction with different gene promoter *cis*-elements mediated by ABA-dependent or ABA-independent signal transduction pathway (see Figure 3).



**Figure 3.** Cereal TFs target gene networks in response to drought through ABA-dependent and -independent pathways. The green arrows show the ABA-independent pathway and the purple arrows show the ABA-dependent pathway.

Most DREB family members such as OsDREB1A [1] and TaDREB1 [41] were found to be activated in ABA-independent pathways. They can enhance the stress tolerance by activating the expression of downstream genes such as late embryogenesis abundant (*LEA*) genes driven

by DRE/CRT *cis*-element. *LEA* genes, such as *COR15A* and *rd29A* (*Cor78*), are also designated as dehydrins (DHNs) or cold-responsive (COR) genes. Their expression products participate in cells protection from stresses by enhancing the membrane stability and correction of protein folding [13]. However, recent studies showed that some members of DREB also participate in ABA-dependent pathways. Wang et al. [3] found that the overexpression of *OsDREB1F* in transgenic rice results in the expression of ABA-induced genes *rd29B* and *RAB18*, whose promoters only contain an AREB element that cannot be recognized by DREB TFs. So far, there is no explanation on illuminating the way for *OsDREB1F* activating the expression of these two genes. The hypothesis is that *OsDREB1F* might interact with bZIP family members that can bind with an AREB element and modulate the transcription of *rd29B* and *RAB18* [3]. The same is true for *ZmDREB2A* [110] and the mechanism is still not clear. Besides, *ZmDREB2A* was identified to activate some downstream genes encoding detoxification enzymes that can protect cells from ROS. However, these genes do not contain a DRE/CRT element. Hence, it is possible that *ZmDREB2A* indirectly affects these genes' expression [110]. Besides, DREBs in different species might have different preference in regulating the expression of downstream genes with different core elements in their promoters. The rice *OsDREB1A*, e.g., prefers to interact with the CRT/DRE core element GCCGAC of genes such as *cor15A*, *rd29A*, and *rd17* instead of core element ACCGAC, while the *Arabidopsis* DREB1A and maize *ZmDREB1A* have equal competition for recognizing core element GCCGAC and core element ACCGAC in the downstream genes [1, 11].

In the WRKY family, more than 10 TFs isolated from rice were found to co-express under drought and cold stresses, but the downstream genes were still not determined [81]. TaWRKY44 from wheat was identified to recognize the core element (TTGACC/TTAACC) in the promoter region of downstream genes and up-regulate genes encoding antioxidant enzymes such as NtSOD, stress-defensive proteins such as NtERD10C, and lipid-transfer proteins such as NtLTP1 to increase plants survival rate in drought. Thus, TF TaWRKY44 participates in regulating antioxidant enzyme activity and decreasing the ROS levels in order to prevent oxidative damage in plant cells [111].

Members of the MYB family regulate the expression level of different target genes involved in the ABA-dependent and independent pathways. The overexpression of *OsMYB48-1* was found to regulate genes such as *OsPP2C68*, *RAB21*, and *OsNCED4*, respectively, involving in ABA early signaling, late response, and the ABA synthesis pathway, contributing to increased drought tolerance under water deficiency [72]. In transgenic *Arabidopsis*, the overexpression of *OsMYB3R-2* increased the expression level of the downstream genes *DREB2A*, *COR15A*, and *RCI2A* and enhanced the plants adaptation to abiotic stresses [20]. Besides, TaMYB30-B was found to induce the expression of stress inducible genes *rd29A* and *ERD1*, involved in the ABA-independent pathway [7].

Members of the bZIP family were also found to regulate downstream gene transcription through the ABA-dependent and independent pathway. OsbZIP52 was suggested to bind to G-box *cis*-elements and down-regulate genes such as *OsLEA3* and *OsTPP* that can improve drought or cold tolerance in rice via the ABA-independent pathway [24]. The expression of

downstream genes *LEA3* and *Rab16* was activated by the transcription factor OsbZIP12 under water deficiency mediated by the ABA synthesis pathway [34].

Most TFs in the NAC family participate in the ABA synthesis pathway. OsNAC5 and OsNAC6 can recognize a core sequence (CACG) of the downstream gene *OsLEA3* and regulate the gene expression that changed root structure and resulted in higher drought tolerance through the ABA signaling pathway [35]. OsNAC6 also participates in up-regulating the transcription of genes encoding peroxidase, which can catalyze a series of oxidative reactions [33]. Some TFs even participate in both ABA-dependent and independent pathways. For example, TaNAC67 was found to up-regulate 10 abiotic stress responsive genes such as *rd29A* and *rd29B*, which were separately related to ABA-independent and -dependent pathways and four ABA synthesis/responsive genes such as *ABI1* [54], thereby improving stress tolerance in plants.

## 5. Homo- and hetero-dimerization of TFs

Homo- and hetero-dimerization of TFs plays an important role in certain cases and is considered as a pre-requisite for binding of DNA *cis*-elements. Formation of homo- and hetero-dimers plays a further function in modulating the DNA-binding specificity of TFs. Inability to form a dimeric complex may absolutely abolish the DNA binding ability of certain classes of TFs. The high complexity in the selection of hetero-dimerization partners and inability of some TFs to homo-dimerize but hetero-dimerize suggests that homo- and hetero-dimerization of TFs are not random processes, but that specific interactions between monomeric TFs forms are preferred. Hence, dimerization is likely to fulfill specific functions in gene regulation.

The dimerization ability of NAC proteins has been localized to the NAC domain [112, 113]. The residues in the highly conserved NAC domain are involved in the dimer contact and consist of hydrophobic interactions, a twisted anti parallel  $\beta$ -sheet sandwiched between two helices and two prominent salt bridges formed by the conserved arginine and glutamate [114, 115]. Experimental data suggest that NAC TFs are capable of forming both homo- and hetero-dimers. The NAC domain of NAC1 [116] and ANACO19 [113, 114] were shown to form homo-dimers. The NAC domains of OsNAC5 were shown to interact with the NAC domains of OsNAC5, OsNAC6, and SNAC1, generating both homo- and hetero-dimeric complexes. BnNAC14, a *Brassica napus* NAC protein, was shown to form hetero-dimers with BnNAC5-8, BnNAC485, and BnNAC3, but not homo-dimers. Mutational and deletion studies suggested that conserved NAC domains, in particular, the amino acids in close proximity to both the amino and carboxy-terminals, are necessary for mediating the formation of homo- or hetero-dimers [106, 112, 117].

Dimerization of the bZIP class of TFs is mediated by leucine zipper motifs, i.e., non-canonical repeats of leucine or other hydrophobic amino acid residues creating an amphipathic  $\alpha$ -helix. The electrostatic attraction and repulsion of the polar residues situated next to the hydrophobic residues enables the formation and stabilization of dimers [118, 119]. Homo-dimeric rice OsbZIP71 is capable of exchanging its subunit to form hetero-dimers with members of the Group-C, in particular, with OsbZIP15, OsbZIP20, OsbZIP33, and OsbZIP88, suggesting a



possible role of hetero-dimerization in efficient binding to *cis*-elements on promoters of target genes [25]. A member of Group-A, the G-box-binding factor AtGBF4, interacts with the Group-G AtGBF1 and the Group-H AtGBF2. Similarly, the members of *Arabidopsis* Group-E, bZIP34 and bZIP61, form hetero-dimers with bZIP51 of Group-I and bZIP43 of Group-S, but none of these TFs belonging to Groups E and I can form homo-dimers due to electrostatic violations in the leucine zipper regions [15, 120, 121]. These data suggest that dimerization between members of within and between groups of bZIP TFs is highly specific and acts as a crucial mechanism to modulate the affinity for *cis*-elements and function of TFs.

In HD-Zips, the leucine zipper that is immediately downstream of the helical domain enables dimerization of HD-Zip TFs, which is a pre-requisite for DNA binding. The HD-Zip leucine zipper is a canonical repeat of leucine amino acid at every seventh residue creating an amphipathic  $\alpha$ -helix, which forms a coiled coil structure during dimerization. Formation of hydrophobic interface and complementary charge interactions by the residues present in the coiled coil structure permit or inhibit the formation of dimers from monomeric HD-Zip TFs [105]. *In-vitro* studies have shown that dimerization of HD-Zip is a pre-requisite for DNA binding, and it is assumed that members of HD-Zip Class I and Class II families form hetero-dimers exclusively with other members of their own family [105, 122–124].

Though there is clear evidence for homo- and hetero-dimerization of WRKY proteins, the extent to which they form a functional dimer is unknown and yet to be determined. Of the seven WRKY subclasses, interaction between members of four WRKY class TFs have been experimentally demonstrated. In Group IIa WRKY TFs, dimerization is mediated by a canonical leucine zipper sequence, whereas in members belonging to other Group II and Group III subclasses, presence of leucine/isoleucine/valine residues at approximate seven-residue intervals at their N-termini form an amphipathic  $\alpha$  helices similar to the secondary structure of a basic leucine zipper and mediate dimerization [125]. It is suggested that these potential leucine zipper sequences might mediate the formation of homo- and hetero-dimers within and between members of different subclasses of WRKY TFs. For example, *Arabidopsis* WRKY TFs belonging to the Group IIa, AtWRKY18, AtWRKY40, and AtWRKY60, form homo- and hetero-dimers [126]. Similarly AtWRKY30 interacted with AtWRKY53, AtWRKY54, and AtWRKY70 and formed hetero-dimers through leucine zipper motifs present at the N-termini of the subclass of WRKY TFs [127]. Interaction between different subclasses was observed in rice. OsWRKY71, a Group IIa WRKY TF, interacted not only with itself, but also with a Group IIId WRKY protein, OsWRKY51 [128]. Formation of homo- and hetero-dimer complexes between different WRKY TFs can have positive or negative effects on their DNA binding activities.

Formation of homo- and hetero-dimers offers an additional large combinatorial flexibility in the regulation of transcription. Performing an accurate analysis and developing a deeper understanding of roles of TFs in various biological processes will require the knowledge of other interacting partners, downstream genes, and location of expression in plant organs along with mechanism of homo- and hetero-dimerization of particular TFs. Thus, it may prove difficult to attempt to make informative conclusions about the roles of specific TFs on the basis of their singular overexpression without this level of knowledge.



## 6. Conclusions

TFs play a vital role in regulating gene transcription through different signal pathways to enable plants to adapt to harsh environments and abiotic stresses such as drought. Those TFs can recognize and interact with specific *cis*-elements of target genes via DBDs. Some TFs can up-/down-regulate downstream gene transcription, which encodes a subset of TFs integrated in plant hormone signaling pathways, forming a complex hierarchic regulatory network. ABA, JA, and GA, the main plant hormones, act as key regulators in balancing plant growth and abiotic stress response. TFs, as the node of the cellular stress network and growth process, function as the interface of different phytohormone signal transduction pathways. A further layer of complexity is the formation of homo- and hetero-dimers, playing an important role in regulating DNA-binding specificity of TFs. These networks of signal pathways are regulating the activity of stress response TFs and other stress-relative genes, which in turn modulate physiological functions, such as stomatal movement, cuticle formation, and carbohydrate and lipid metabolism, to limit water loss and adapt to drought conditions. However, the hierarchy of TF interactions, the downstream genes' network, the interaction mechanism of the signal transduction pathways, and the protein-protein dimerization are not fully explored and still need more effort to be understood. More knowledge about plant protection system in hostile environments will help to find new tools for enhancing the plants to adapt to abiotic stresses.

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# Histone Methylation - A Cornerstone for Plant Responses to Environmental Stresses?

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Additional information is available at the end of the chapter

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## Abstract

Plants are sessile organisms and, as such, their survival relies on their ability to respond quickly all along their life cycle to any kind of environmental stimuli, including abiotic and biotic stresses. In this respect, plants have developed efficient mechanisms of protection and/or adaptation to minimize deleterious effects of stress on their growth and development. In a stress type-dependent manner, external signals are firstly sensed. This step is then followed by the activation of particular signalling pathways, resulting ultimately in the rapid and specific modulation of the plant transcriptome. Currently, transcriptional regulation is considered as a central process in the build-up of plant responses to both abiotic and biotic stresses. Among mechanisms involved in transcriptional regulation, the combined effect of different histone tail post-translational modifications (PTMs; e.g. acetylation and methylation) through the activity of particular histone-modifying enzymes can lead to changes in the local chromatin structure environment and hence the underlying DNA accessibility.

By focusing on histone lysine methylation, in this chapter we highlight our current understanding of the transcriptional roles played by chromatin-remodelling mechanisms in regulating plant response/adaptation to different biotic and abiotic stresses. Based on recent advances, we further discuss the stability and transmission of such methylation marks to subsequent generations, with the underlying idea of an epigenetically based transcriptional memory of stresses in plants.

**Keywords:** Histone methylation and demethylation, histone methyltransferases and demethylases, biotic and abiotic stresses

## 1. Introduction

Stress, as we currently think of it, is a highly subjective phenomenon defined as a state of threatened homeostasis. Depending on their nature, external stresses are usually divided into biotic (i.e. herbivorous insects and pathogens such as fungi, bacteria and viruses) or abiotic (i.e. including, among others, high or low temperature, submergence or drought and salinity). During their lifetime, all living organisms inevitably and constantly face all sorts of environmental stresses that often occur suddenly and/or simultaneously. Classically, different strategies can be applied to minimize deleterious effects of stresses, such as resistance, tolerance, avoidance or escape. Being sessile, plants cannot escape and are therefore more prone to the deleterious effect of unfavourable environmental growth conditions. Because responses are critical to ensure their survival, plants have developed specific and efficient strategies that allow them to precisely perceive different environmental stresses and respond and/or adapt to them [1, 2]. In addition to preformed defence traits, plants have evolved inducible defence strategies. Indeed, upon perception, each stress will raise a complex and more or less specific repertoire of cellular and molecular responses implemented by the plant to minimize or prevent damage. Particularly, the stimulation of a given stress-signalling pathway after pathogen detection will be integrated into the plant cell nucleus through a set of regulatory transcription factor cascades, which prioritizes defence over growth-related cellular functions, while conserving enough valuable resources for survival and reproduction [3, 4]. Supporting the idea that the capacity of a plant to rapidly reprogramme its gene expression at the transcriptional level is an essential and common component of all plant response strategies to stress and disturbance; more than 1,000 transcription factors were found to be involved in stress responses [5, 6]. Because eukaryotic genes function in the context of chromatin, modifications and remodelling of the chromatin configuration from permissive for transcription to restrictive, and vice versa, may be an integral part of mechanisms involved in this vital transcriptional reprogramming. In this chapter, we review and discuss the current knowledge about the functional impact of chromatin changes on the transcriptional regulation of genes under different stress conditions, with particular emphasis on histone methylation/demethylation.

## 2. Chromatin structure and histone methylation/demethylation

In eukaryotes, genomic DNA in the cell nucleus is packaged in a complex and evolutionarily conserved structure named chromatin, with nucleosome as the basic unit. The nucleosome complex contains about 160–241 base pairs (bp) of DNA, a nucleosome core particle and the H1 linker histone. The nucleosome core particle is composed of an octamer of core histones, consisting of two H3–H4 dimers associated with two H2A–H2B dimers. About 146 bp of DNA is wrapped in ~1.65 negatively supercoiled circles around the histone octamer, while the linker DNA associated with H1 varies in length from 8 to 114 bp [7]. At first sight, the chromatin as it is described appears as a barrier, restricting the access of all kinds of enzymes that process the DNA. However, nucleosomes are not merely static but highly dynamic entities. Indeed,



nucleosomes can be moved, stabilized/destabilized, disassembled/reassembled at particular genome locations in response to specific environmental signals or developmental cues [8]. This dynamic leads to a wide range of chromatin condensation states modulating the DNA accessibility, with euchromatin, being relaxed, and heterochromatin, being compacted. Therefore, in eukaryotic cells, an intimate connection exists between the structural organization of the genome and its functioning. For this reason, the level of chromatin condensation is directly related to all aspects of DNA metabolism, thus playing a major role in regulating transcription, DNA replication, DNA repair, recombination, transposition and chromosome segregation. In plants, changes in the chromatin structure were reported to affect various biological processes such as root growth, flowering, organogenesis, gametophyte or embryo formation [9–11].

In the nucleosome core particle, histones H2A, H2B, H3 and H4 possess two common regions, a histone-fold domain and a histone tail. The histone-fold domain is the most conserved region and the main element of histone dimerization [12]. The tail protrudes from the nucleosome core particle and is more variable and unstructured than the fold [13]. All four core histones have an N-terminal tail domain, but only histone H2A has an additional long C-terminal tail. Histone tails are extremely basic due to their particularly high content in basic amino acid residues, such as lysine and arginine [14]. Resulting positive charges allow them to closely associate with the negatively charged nucleosomal DNA through electrostatic interactions [15]. In addition, histone tails, especially N-terminal ones, may undergo diverse types of post-translational modifications such as acetylation or methylation. The great diversity of these modifications as well as the high number of amino acid residues that can be modified within histone tails, and the correlation between these modifications and various nuclear processes, lead to the hypothesis that the specific combination of histone modifications constitute a histone ‘code’ [16].

Technically, these histone marks can be localized by chromatin immunoprecipitation (ChIP) using specific antibodies against the modification [17]. Briefly, protein–DNA interactions are stabilized by cross-linking with formaldehyde; chromatin is sheared into small pieces to facilitate analysis and then immunoprecipitated using an antibody raised against a specific histone modification. Following enrichment, cross links are reversed to release DNA, which is then quantified by polymerase chain reaction (PCR) to measure the relative amount of the specific histone mark on selected plant genes. ChIP can also be combined with microarray hybridization (ChIP-chip) or high-throughput sequencing (ChIP-Seq), allowing the genome-wide discovery of DNA–histone modification interactions.

Methylation is the most abundant one compared with other histone PTMs. It can occur at both lysine (K) and arginine (R) residues of core histone tails. Further extending the indexing potential of this modification, mono-, di- and trimethylation of lysine and mono- and dimethylation (symmetric or asymmetric) of arginine are common at N-terminal tails of H2A, H2B, H3 and H4. Although histone acetylation is generally associated with active gene transcription, histone methylation can be associated with either active or silent gene expression, depending upon the histone, the methylated residue or the level (mono-, di- or tri-) of methylation. In *Arabidopsis*, genome-wide analyses revealed that trimethylations of H3K4 and H3K36

(H3K4me3 and H3K36me3) are generally enriched at actively transcribed genes, whereas H3K27me3 is associated with repressed genes and H3K9me2 and H4K20me1 are enriched at constitutive heterochromatin and silenced transposons [18]. For histone arginine methylation, a definitive role has not yet been clearly established. However, because the level of symmetric H3R2me2 and H4R3me2 was negatively correlated with the level of H3K4me3, a well-known mark reflecting active transcription, high levels of H3R2me2 and H4R3me2 are thought to cause transcriptional repression [19–21]. In contrast, asymmetric H4R3me2 was associated with gene activation [22, 23].

Histone methylation is relatively stable and can be established on lysine and arginine by two distinct families of enzymes, the histone lysine methyltransferases (HKMTs), all containing the evolutionary conserved catalytic SET domain in plants [24], and the protein arginine methyltransferases (PRMTs) [25], respectively. As a counterpart, methyl groups on histone can also be removed by at least two evolutionarily conserved classes of histone demethylases, the lysine-specific demethylase1 (LSD1) type and the Jumonji C (JmjC) domain-containing demethylases [26]. Histone methyltransferases and demethylases are well conserved in angiosperms and have been identified and classified on the basis of phylogenetic analyses and domain organization in several plants, including *Arabidopsis*, maize, tomato, rice, grapevine and *Brassica rapa*, [27–32]. However, cellular and molecular functions of many of these modifiers have not yet been addressed.

Although histone acetylation can directly modulate the chromatin structure, arginine and lysine methylation of histone tails can promote or prevent the docking of key transcriptional effector molecules, named readers, needed to ‘translate’ the code in order to determine the functional and structural outcome of the corresponding PTMs. Just as there are a large number of PTMs on histone tails, there are also numerous protein domains that recognize and bind to particular PTMs on these tails. For example, PTM-recognition domains such as plant homeo-domain (PHD) fingers, chromodomains and Tudor domains all recognize methylated lysine residues [33].

### 3. Histone methylation changes associated with biotic stress conditions

Biotic stress is the result of the damage done to plants by insects or pathogens, such as bacteria or fungi. Plant pathogens are generally divided into two distinct categories: biotrophs, which colonize living plant tissue and obtain nutrients from living host cells, and necrotrophs, which depend on dead host tissue for nutrients and reproduction. To fend off pathogens with different infection strategies, plants have evolved complex defence mechanisms. Classically, the pathogen-sensing machinery induces signalling cascades that promote the accumulation of hormones such as salicylic acid (SA) or jasmonic acid (JA)/ethylene (ET) [34]. These hormones then orchestrate the overall plant defence reaction locally and systemically by inducing the transcriptional activation of defence genes through an intricate signalling network. In this part, we highlight recent examples illustrating how histone methylations condition major steps leading to immunity, ranging from initial pathogen perception to hormonal homeostasis changes for antimicrobial effector expression.

### 3.1. Histone methylation/demethylation in the defence against biotrophic pathogens

The phytohormone SA plays an important role in plant defence, from the induction of pathogen resistance (*PR*) genes against biotrophic bacteria (e.g. *Pseudomonas syringae*) to the establishment of systemic acquired resistance (SAR) [35]. Several studies suggested that the SA signalling pathway is notably controlled by histone methylation. Under normal growth conditions, *Arabidopsis* mutants for *SNI1* (*Suppressor of NPR1, Inducible*), a negative regulator of SAR required to dampen the basal expression of *PR* genes, presented an increased H3K4me2 on *PR1* [36]. Rather than being a constitutive mark of transcription, H3K4me2 was proposed to be involved in the fine-tuning of tissue-specific expression [37]. Using the functional SA-analogue S-methyl benzo [1,2,3] thiadiazole-7-carbothioate (BTH), an increased level of H3K4me2 on *PR1* was observed in wild-type plants 48 h after treatment and was not detected in mutants. Interestingly, when expressed in yeast, *SNI1* also repressed transcription, suggesting a highly conserved mechanism of transcriptional repression. These results together with the structural similarity of *SNI1* with armadillo repeat (ARM) proteins (i.e. a motif known to mediate protein–protein interactions) imply that *SNI1* may form a scaffold for interaction with proteins that modulates the chromatin structure of *PR* genes, thus repressing their transcription. In addition, the presence of H3K4me2 detected on *PR1* before induction suggested that this mark is readily in place, providing the appropriate chromatin configuration for the efficient induction of *PR1* upon need. Using a similar approach, Alvarez-Venegas et al. [38] reported no significant changes in levels of H3K4me2 and H3K4me3 on *PR1* 24 h after the SA treatment [38]. This discrepancy may reflect differences in experimental conditions. Indeed, the action of the so-called ‘SA-analog’ BTH on gene transcription is significantly broader than the action of SA itself [39]. Moreover, samplings were performed 48 h versus 24 h after treatment. Together, because the H3K4 methylation increase does not occur immediately after the induction of *PR1*, this mark may not be directly related to the transcriptional induction itself, but later, for the maintenance/reinforcement of *PR1* expression.

The ARABIDOPSIS HOMOLOG OF TRITHORAX (ATX1) is a H3K4 trimethyltransferase providing basal resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*); [40]. Despite being not induced by either *Pst* infection or SA, ATX1 positively and directly regulates the expression of the transcription factor WRKY70 through H3K4 trimethylation at the WRKY70 promoter. In addition, *atx1* mutant shows induced expression of the JA-inducible *THI1.2* gene and the reduced *PR1* expression without detectable changes in their chromatin, resulting in impaired resistance to *Pst* infection. Since the transcriptional factor WRKY70 was positioned at the convergence node of the SA and JA signalling pathways, activating the SA-responsive *PR1* gene and repressing the JA inducible genes [41], ATX1 was proposed to indirectly regulate *PR1* and *THI1.2* through WRKY70. SET DOMAIN GROUP 8 (SDG8), another HKMT encoding the major *Arabidopsis* H3K36 di- and trimethyltransferase [42], was also involved in the plant-defence against *Pst*, but it was more upstream than ATX1 [43]. Indeed, SDG8 sustains the basal transcription of particular *R* genes (*RPM1* or *LAZ5*) by maintaining a basal level of H3K36me3, another histone mark tightly associated with active transcription. SDG8 is also required for the transcriptional induction of these *R* genes upon BTH treatment or *Pst* inoculation. However, this induction occurs without any detectable increase of H3K36me3. Therefore, in resting

plants, SDG8 may establish a 'permissive' chromatin structure at some *R* genes by methylating H3K36, thus ensuring their basal expression and their transcriptional inducibility upon need. Similarly as *atx1* and *sdg8* mutants, loss-of-function mutants for the putative HKMT SDG7 were also found to be more sensitive to *Pst* infection than wild-type plants [44]. The expression of other *R* genes seems to be under the control of histone methylation. Indeed, enhanced downy mildew 2 (EDM2) impacts disease resistance by controlling levels of H3K9me2 at an alternative polyadenylation site in the immune receptor gene *RPP7*, thus regulating the balance between full-length *RPP7* transcripts and prematurely polyadenylated transcripts, which do not encode the *RPP7* immune receptor [45, 46]. EDM2, as an epigenetic 'reader', contains two stretches of atypical PHD-finger motifs known to dock specifically several forms of methylated or unmethylated lysine residues on histones [47]. Besides this, EDM2 was also proposed to cooperate within a large protein complex with EMSY-like (AtEML) members, harbouring an Agenet domain related to the Tudor domain family of epigenetic 'readers' [48].

Apart from *Arabidopsis*, little is known about the regulatory role of histone methylation in the defence against pathogen attack in other plant species. In rice, the JmjC protein gene *JMJ705* encoding a histone lysine demethylase that specifically reverses H3K27me2/3 was found induced during infection with the bacterial pathogen *Xanthomonas oryzae*. *JMJ705* was further involved in the dynamic removal of the basal H3K27me3 over defence-related genes, thereby increasing their basal expression and/or potentiating their higher expression upon biotic stress. Interestingly, the *JMJ705* overexpression resulted in an enhanced resistance to the bacterial pathogen, while its mutation reduces the plant resistance [49].

### 3.2. Histone methylation/demethylation in the defence against necrotrophic pathogens

While to combat biotrophic pathogens the plant activates mainly the SA signalling pathway, the activation of the JA/ET signalling pathway is prominent to mediate defences against necrotrophic pathogens and herbivorous insect attacks [50]. The involvement of histone methylation in the defence against necrotrophic pathogens is far less documented as compared with the defence against biotrophic pathogens. Besides being more susceptible to *Pst* [43], *sdg8* mutants were also reported to be more sensitive to necrotrophic fungal pathogens such as *Alternaria brassicicola* (*Alt*) and *Botrytis cinerea* [51]. This increased susceptibility was the consequence of the inefficient transcriptional induction of different genes along the JA/ET signalling pathway that was correlated with a stably weak level of H3K36me3 at these genes. Inversely, in wild-type plants, H3K36me3 together with gene expression were increased upon *Alt* infection or stimulation with exogenous MeJA. Under resting conditions, a similarly weak level of H3K36me3 was correlated with a reduced basal expression in *sdg8*. On that account, H3K36 methylation was proposed to act as a 'permissive' mark correlated with gene activity and readily in place at a subset of JA/ET signalling-related genes to raise their rapid and efficient transcriptional induction when required [52]. Interestingly, a stable and very low level of H3K27me3 was detected in defence effector genes. Because H3K27me3 is often associated with epigenetic silencing [53], this low H3K27me3 level may provide these genes with a reduced probability for undesired silencing, thus participating in the reactivity of plants to pathogen infections.

## 4. Histone methylation changes associated with abiotic stress conditions

Abiotic stresses such as heat, cold, drought, salinity and nutrient deficiency are inherent to every ecosystem and essentially unavoidable. Abiotic stresses are considered the most harmful factors in terms of growth and productivity of crops worldwide [54, 55], especially when they occur in combinations [56]. Here, we summarize and discuss various studies in order to clarify the functional involvement of different histone methylation marks in setting up plant responses to adverse environmental growth conditions.

### 4.1. Histone methylation/demethylation and the plant stress hormone ABA

The phytohormone abscisic acid (ABA) is a crucial signalling molecule playing versatile functions in regulating many developmental processes, including seed dormancy and germination [57, 58]. ABA also plays a pivotal role in adaptive stress processes, integrating both biotic and abiotic environmental constraints in a complex network of interacting pathways with crosstalks at different levels [59–61]. Currently, ABA is considered as a global regulator of stress responses that can dominantly control the switch in priority between the responses to biotic or abiotic stress, allowing plants to respond to the most severe threat [62].

The transition from heterotrophic to autotrophic development at the post-germinative stage (i.e. embryonic state) is highly vulnerable to osmotic stress [63]. During a period of osmotic stress, ABA promotes the expression of transcription factors such as *ABI3* and *ABI5*, which in turn delay germination and lead to osmotolerance and survival [64]. In *Arabidopsis*, mutation in *PICKLE* (*PKL*), encoding a putative chromatin modifier, results in increased and abnormally sustained expression of *ABI3* and *ABI5* in response to exogenous ABA treatment. This sustained expression was correlated with reduced levels of H3K9me2 and H3K27me2, two methylation marks found in the chromatin of silent genes [65]. Based on these results, it was suggested that *PKL* might act on *ABI3* and *ABI5* to promote directly or indirectly the formation of a repressed chromatin state through a so-far-unknown mechanism.

In adult plants, the establishment of a response and tolerance to drought stress by ABA has been extensively studied and is well discussed in several outstanding reviews [58, 66]. Briefly, under drought conditions, water stress perception triggers ABA biosynthesis and increased tissue ABA accumulation, resulting in stomatal closure and reduced transpiration. Among major enzymes involved in the ABA biosynthesis pathway, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (*NCED3*) is thought to be the rate-limiting enzyme [67]. In *Arabidopsis*, the increased transcription of *NCED3* upon dehydration was correlated with the binding of the HKMT *ATX1* and the increased level of H3K4me3 at *NCED3* [68]. Therefore, the loss-of-function *atx1* mutant showed less tolerance to dehydration, notably because of the lower enrichment of RNA Polymerase II (RNAPII) and H3K4me3 at *NCED3* under stress. *ATX1*-modified H3K4me3 may thus have an important function in the transcriptional regulation of *NCED3*. However, it is still unclear whether this function is directly linked with the transcriptional induction or is more related to the reinforcement of the increased transcription upon stress perception.

## 4.2. Histone methylation/demethylation in response to water stresses

Water stresses including drought or submergence are major environmental factors limiting plant growth and crop productivity worldwide [69, 70]. Consequently, plants have evolved a variety of biochemical and physiological mechanisms to respond/adapt to these stresses [71, 72]. In the following section, we distinctly address the involvement of histone methylation in responses to drought and submergence.

### 4.2.1. Drought stress

Using *Arabidopsis*, the molecular response to water deficit was found to rely notably on the transcriptional regulation of stress-inducible genes with products thought to function in drought tolerance and response [73]. Using ChIP analyses in 15-day-old *Arabidopsis* seedlings, the level of the active mark H3K4me3 was found gradually enriched in response to dehydration stress, preferentially on the coding region of four drought-inducible genes (*RD29A*, *RD29B*, *RD20* and *RAP2.4*), and was correlated with their upregulation [74]. Consistent with this result, *ATX1* was reported to be required for the efficient transcriptional induction of *RD29A* and *RD29B* during a dehydration stress response in an ABA-independent manner [68]. Also, for unknown reasons, it is worth noting that upon exogenous ABA treatment the transcriptional induction of *RD29A* and *RD29B* was stronger in *atx1* mutants than in wild-type plants (their basal transcript levels were lower in *atx1* than in wild-type, while their transcript levels upon ABA treatment were identical [68]). Next, for the ABA-dependent *RD29A* and ABA-independent *RAP2.4* genes, a time lag was observed between their transcriptional induction and the increase in H3K4me3 [74]. Based on these findings and the rapid saturation in RNAPII enrichment compared with H3K4me3 (i.e. already saturated 1 h after stress exposure for RNAPII, while H3K4me3 was still increasing up to 5 h), Kim et al. [74] concluded that the H3K4me3 enrichment may be established, gradually, in response to drought stress after full transcriptional activation of *RD29A* and *RAP2.4*. Because the timing of H3K4me3 enrichment followed subsequent to the RNAPII enrichment, H3K4me3 might be dispensable for the initiation of transcription. Finally, the gradual increase of H3K4me3 further indicates that the longer the stress lasts, the more H3K4me3 will be enriched, suggesting that the epigenetic responsiveness must depend on the intensity of a stress [75].

In a similar approach but using ChIP-Seq, van Dijk et al. [76] established the whole-genome distribution patterns of H3K4me1, H3K4me2 and H3K4me3 in 4-week-old rosette *Arabidopsis* leaves under dehydration stress conditions. They also observed a strong correlation between H3K4me3 abundance and transcripts levels from responding genes. Indeed, among the most strongly downregulated genes, an increase in H3K4me1 and a decrease in H3K4me3 were detected, suggesting the involvement of a histone demethylase in modulating the expression of a subset of stress-responsive genes. Supporting this finding, a putative PKDM7 subfamily-like H3K4 demethylase homologue and two putative demethylase enzymes containing a JmjC domain were found to be drought-inducible in two barley cultivars and in young peanut plants, respectively [77, 78]. Surprisingly, in contrast to the classical genome-wide H3K4me3 enrichment around the transcriptional start site observed in all other eukaryotes [79], H3K4me3 displayed a broader distribution on dehydration and ABA-inducible



genes. Such an unusual profile may reflect a function not strictly related to transcription initiation, as will be discussed later in the stress memory section.

Through a genome-wide approach in rice seedling, Zong et al. [80] also uncovered a weak but positive correlation between H3K4me3 enrichment and the transcript level of some drought-responsive genes under drought stress. This correlation was extended to many genes involved in stress-related metabolite and hormone signalling pathways, further supporting the role played by H3K4me3 in the stress response [80]. However, because H3K4me3 is not the only histone mark for gene activation, this weak correlation may reflect that other active histone marks may also play important roles in regulating gene expression in response to stress in rice. Although these large data sets have provided much information on drought responses in rice, more detailed analyses will be required to elucidate whether the observed variations in H3K4 methylation are a cause or a consequence of the transcriptional changes triggered by water stress. Moreover, identifying key histone modification enzymes is indispensable to better understand the transcriptional regulatory network of the abiotic stress response.

#### 4.2.2. Submergence

Submergence is a complex stress that encompasses many changes in environmental factors, including light intensity, pH and dissolved oxygen concentration. Alcoholic fermentation is important for the survival of plants especially under anaerobic environments [81]. In rice, *alcohol dehydrogenase 1* (*ADH1*) and *pyruvate decarboxylase 1* (*PDC1*) genes are involved in this anaerobic metabolism and their expression is reversibly induced (i.e. activated upon submergence and repressed upon re-aeration) [82]. Using these two genes as a model, Tsuji et al. [83] observed that the level of H3K4 methylation, specifically at both the 5'- and 3'-coding regions of *ADH1* and *PDC1*, was changed from a dimethylated state to a trimethylated state upon their transcriptional upregulation in response to submergence. This change was reverted back to its initial level following re-aeration, indicating that in this particular case, H3K4me3 does not serve as a memory mark of a prior transcriptional activity. Similarly to drought, these results highlight the dynamic and reversible change of histone H3K4 methylation at stress-related genes in response to the occurrence and disappearance of a stress.

### 4.3. Histone methylation/demethylation in response to salt stress

Salinity is also a serious factor affecting plants in several ways (i.e. water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, genotoxicity, reduction of cell division and expansion), thus limiting plant growth, development and survival [84]. In *Arabidopsis*, a NaCl or ABA treatment has been shown to induce the transcription of a range of abiotic stress-responsive genes (*ABI1*, *ABI2*, *KAT1*, *KAT2*, *DREB2A*, *RD29A* and *RD29B*). Using ChIP, this induction was found significantly correlated with an increase in the active mark H3K4me3 and a decrease in the repressive mark H3K9me2 [85]. Also, suggesting a link between different histone PTMs, mutations in the histone deacetylase HDA6 partially suppressed the H3K4me3 increase observed in response to stress, while the H3K9me2 decrease was not affected. Since histone acetylation and H3K4 trimethylation are often associated with gene activation [86], the repressive function of HDA6 may

suggest that acetylation on histone H3 is required for K4 methylation to occur [87]. Unfortunately, because ChIP experiments were not normalised against the total H3 density, it is difficult to attribute the decrease in H3K9me2 to either the active removal of methyl groups by a histone demethylases or the active removal of nucleosome in response to a stress. Supporting the second possibility, the nucleosome density of two other drought stress-inducible genes (*RD20* and *RAP2.4*) was found to gradually decrease in response to drought stress [74].

In soya bean and in response to a high NaCl concentration, these histone methylation marks were also found altered at some salinity-induced transcription factors (i.e. MYB, b-ZIP and AP2/DREB family members) that were primarily identified by microarray analysis [88]. For some genes, their transcriptional induction was correlated with an increased level of histone acetylation and H3K4me3, accompanied or not with a reduced level of DNA methylation and H3K9me2 in various parts of the promoter or coding regions. For other genes, DNA methylation had no influence on histone methylation. This work perfectly reflects the heterogeneity of the effect of salinity on histone methylation and DNA methylation, and supports the role(s) of histone methylation changes in the expression of some transcription factors important for salinity tolerance.

As mentioned above, H3K4me3 was found to be involved in the transcriptional induction of stress-responsive gene upon salt stress exposure. In plants, the JmjC-domain-containing histone demethylases JMJ14, JMJ15 and JMJ18 have been reported to display an H3K4me2/3 demethylase activity as well as to regulate diverse aspects of chromatin function and development [89–95]. Recently, the overexpression of *JMJ15* was reported to preferentially down-regulate many stress-related genes preferentially marked by H3K4me2/3 and to enhance salt stress tolerance [96]. In contrast, the loss-of-function mutant was more sensitive to salt. Despite the fact that an increased JMJ15 level may regulate stress-responsive gene transcription programmes in *Arabidopsis*, the role of H3K4me3 resetting in these processes is still very elusive.

Besides histone lysine methylation, arginine methylation was also involved in establishing the transcriptional response to salt stress. The protein arginine methyltransferase 5 (PRMT5), also named Shk1 kinase-binding protein1 (SKB1), is a type II methyltransferase that catalyses symmetric H4R3 dimethylation, a repressive mark known to promote flowering through the repression of the floral repressor *FLOWERING LOCUS C (FLC)* in *Arabidopsis* [20, 97]. Interestingly, gain-of-function *SKB1* mutants showed an enhanced salt stress tolerance and sensitivity to ABA [98]. As a consequence of PRMT5 disassociating from chromatin, the H4R3me2 level at stress-responsive genes was reduced during salt stress, resulting in their induced expression. Suggesting an additional function of PRMT5 on non-histone proteins, the methylation level of the U6 small nuclear ribonucleoprotein Sm-like4 (LSM4, a core protein of the spliceosome) was increased in response to salt stress and ABA. Since splicing defects were observed in the *prmt5/skb1* and *lsm4* mutants, with both of them being hypersensitive to salt stress, authors proposed that PRMT5 might mediate plant development and salt response by altering the methylation status of H4R3me2 and LSM4, linking transcriptional regulation to pre-mRNA splicing [98].



#### 4.4. Histone methylation/demethylation in response to temperature

In plants, temperature stresses are classically classified into different types according to temperature exposure, which may be warm, high, chilling or freezing temperature. Due to global warming and because temperature stress greatly affects plant growth and development, immunity and circadian rhythm, and poses a serious threat to the global food supply, the genetic mechanisms of plant responses to heat have been well studied. Plants exposed to temperature stresses modulate the transcription of a large number of genes involved in distinct biochemical and physiological response pathways and networks of phytohormones or secondary metabolites, ultimately leading to increased tolerance to hazardous temperature stresses [99–102]. The role played by histone methylation during the plant response to a heat or a cold stress is discussed separately hereafter.

##### 4.4.1. Heat stress

Heat stress during seed development decreases the seed size in many cereals, resulting in severe yield losses [103, 104]. In rice, a molecular mechanism involving the putative rice polycomb repressive complex 2 (PRC2) gene *Fertilization-Independent Endosperm1* (*OsFIE1*) was suggested as being a potential key component involved in regulating the thermal sensitivity of seed enlargement during endosperm development [105]. When developing seeds were exposed to a heat stress, both DNA methylation and H3K9 methylation were reduced on *OsFIE1* resulting in its derepression. Under heat stress, syncytial stage-specific MADS-box genes involved in seed size regulation were precociously repressed, due to the increased deposition of H3K27me3 silencing marks by the PRC2 complex [105]. In the unicellular green alga *Chlamydomonas reinhardtii*, histone modification was also affected by heat stress [106]. Indeed, after heat stress, the level of H3K4me1 was found decreased and the level of histone acetylation increased at promoter regions of active genes compared with inactive regions. As a hypothesis, authors proposed that upon heat stress, the heat shock transcription factor HSF1 might promote chromatin remodelling and RNAPII recruitment for transcription initiation/elongation [106]. Finally, while addressing molecular mechanisms of the response of cotton anthers to high temperature, two jmjC domain-containing genes, putatively involved in histone demethylation, were found significantly repressed during anther development under heat stress [107].

Because euchromatin is gene rich and usually transcriptionally active, investigation about the role of histone methylation in temperature stress acclimation was largely centred on euchromatin-associated coding regions. Focusing on the transcriptionally silent heterochromatin, mainly constituted of repetitive DNA sequences, some works demonstrated the transcriptional activation of normally silent transposable element embedded within heterochromatic regions under stress conditions [108]. Intriguingly, such activation under heat stress can occur without alteration of DNA methylation and with only minor changes in both H3K9me2 and H3K4me3 [109, 110]. In summary, these works suggest that temperature stress-mediated transcription of tandem-repeat elements might play a vital role in the adaptation of plants to temperature stimuli, offering an efficient mechanism by which heat or cold could promote the expression of some stress-responsive genes. Upon activation and when inserted into or very close to a

gene, such transposable elements could interfere with the expression of this gene, giving rise to deleterious mutations, genetic instability or positive contribution to gene regulation and adaptation [111].

#### 4.4.2. Cold stress

The increased tolerance of plants to cold is referred as 'cold acclimation'. Cold acclimation differs from vernalization, as the last one requires a long-term exposure to cold temperatures, while cold acclimation can be achieved in a couple of days under non-freezing low temperatures [112]. Locally, histone methylation changes in cold-responsive genes were addressed in *Arabidopsis*. Upon cold stress, the repressive mark H3K27me3 decreased, in both a histone occupancy-dependent (i.e. arising from the lowering nucleosome density) and -independent (i.e. as the result of the activity of a not yet identified histone demethylase) manner, on the cold-responsive genes, cold-regulated 15A (*COR15A*) and galactinol synthase 3 (*ATGOLS3*; [113]). Interestingly, the decrease in H3K27me3 upon stimulation occurred more gradually than their rapid transcriptional induction, so that their activation may not be inhibited by H3K27me3 itself but rather lead to the removal of H3K27me3. Also, while the transcription of *COR15A* and *ATGOLS3* was completely repressed to the initial level upon returning plants to normal growth conditions, the H3K27me3 decrease was maintained. Given that this decrease does not affect the transcriptional induction of *COR15A* and *ATGOLS3* upon re-exposure to cold temperatures, such chromatin change can so far only be view as a 'reminiscence' of a recent transcriptional activity and not as a stress memory implicated in a gene priming process.

In maize during cold stress, changes in histone modifications, including the heterochromatic marks H3K9me2 and DNA methylation, were assessed through a genome-wide approach [114]. The more detailed analysis of the two knob-associated tandem-repetitive sequences, the 180-bp repeat and the 350-bp repeat termed TR-1, demonstrated that their selectively and transiently cold-activated transcription was correlated with a decreased H3K9me2 and DNA methylation, together with an increased H3K9 acetylation. Such cold-induced transcriptional activation of tandem repeats is selective and transient, and the silencing state is recovered as the treatment continues.

## 5. Histone methylation as a memory mark of stress

In animals, the formation of memory immune cells after primary antigen recognition confers long-lasting resistance, resulting in an accelerated and a more effective immune response in case of second exposure. Despite the absence of such memory immune cells, plants often acquire a systemic immunity to further infections after a primary localized infection [115]. This requires the accumulation of the plant hormone SA in systemic tissues and is called systemic acquired resistance (reviewed in [116]). The SAR is also associated with gene priming in systemic tissues, in which defence genes will be expressed more rapidly and robustly in case of a second attack [117]. At the transcriptional level, gene expression is primarily influenced by the chromatin structure, which in turn is controlled partly by processes, often referred to

as ‘epigenetic’ processes, which can be transmitted through mitosis and/or inherited through meiosis [118]. Therefore, chromatin remodelling through histone methylation offers a potential mechanism for short-/long-term stress memory within the lifespan of an individual, referred to as somatic memory, and/or across generations, referred to as transgenerational memory.

### 5.1. Somatic stress memory

In *Arabidopsis*, a priming event, either treatment with BTH or infection with *Pseudomonas syringae* pv. *maculicola*, systematically resulted in an increase in the level of H3K4me2/me3 at defence gene promoters (namely the WRKY transcription factors WRKY6, WRKY29 and WRKY52) that are normally found on active genes, while the genes remain inactive [119]. More interestingly, this increase also occurs in leaves distal to localized foliar infection. Hence, even if the histone-modifying enzyme involved in this process remains unidentified, results from Jaskiewicz et al. [119] clearly suggest that histone methylation might create a ‘memory’ of the primary infection that is associated with an amplified reaction to a second stress stimulus. Further, Luna et al. [120] observed that promoters of SA-inducible PR1, WRKY6 and WRKY53 in the progeny of *Pst*-inoculated *Arabidopsis* plants were enriched with acetylated histone, while the promoter of the JA-inducible gene *PLANT DEFENSIN1.2* (*PDF1.2*) showed an increased level of H3K27me3. For decades, the signalling protein NON EXPRESSOR OF PR1 (NPR1) has been implicated in mediating SAR induction [115] and also the crosstalk between SA- and JA/ET-dependent defence pathways, enabling plants to mount an appropriate defence reaction, depending on the nature of the attacker and the stage of infection [121, 122]. More recently, NPR1 has been proposed to play a critical role in the expression of the transgenerational SAR as progeny from *npr1* failed to develop transgenerational defence phenotypes and failed to present enrichment for H3K27me3 at the *PDF1.2* promoter [120]. Together, these findings suggested that one or more systemic signals are stored as an immune memory on defence-related gene promoters in the form of histone modifications, thus providing the plant with a life-long protection, which can be transmitted to subsequent generations.

Besides being involved in defence priming related to biotic stress, histone methylation was also proposed as a priming strategy against drought. To further explore the functional impact of histone methylation on biotic stress responses in *Arabidopsis*, Kim et al. [123] followed chromatin dynamics of several drought genes (*RD20*, *RD29A* and *AtGOLS2*) and a rehydration-inducible gene (*ProDH*) during drought and rehydration. As previously discussed, a strong correlation was observed between H3K4me3 enrichment (i.e. especially in gene bodies) and transcription for drought genes upon drought [74, 123]. Such a correlation was also detected for the rehydration gene upon rehydration [123]. Suggesting a memory role for H3K4me3, RNAPII rapidly disappeared after rehydration at drought genes, while H3K4me3 was gradually decreased. Concomitantly, by training plants with up to four successive drought treatments, Ding et al. [124] uncovered the existence of two distinct subsets of genes within the dehydration stress-response gene fraction. The ‘non-trainable’ genes (e.g. the ABA-independent *RD29A* and *COR15A*) have repetitively similar transcription rates during each stress treatment, while ‘trainable’ ones (e.g. the ABA-dependent *RD29B* and *RAB18*) increased

the magnitude of their subsequent transcriptional response, relative to their initial stress response. Using ChIP, Ding et al. [124] observed that the H3K4me3 enrichment at 'trainable' genes, especially in gene bodies, was atypically retained from the preceding transcription after rehydration. Even more interestingly, the RNAPII phosphorylated at C-terminal domain (CTD) repeat serine 5 (Ser5P; associated with transcription initiation) was found stalled on these genes as a memory mark from a previously transcribed state. In contrast to 'trainable' genes, the stress-induced H3K4me3 and Ser5P enrichment at 'non-trainable' genes was decreased to its basal level during recovery [124]. Moreover, this transcriptional memory can persist in the absence of inducing signals at least for 5 days, but is lost after 7 days. Supporting a specific role for H3K4me3 in stress memory, other active chromatin marks such as acetylation of histones H3 and H4 were found rapidly increased at drought genes upon stress and decreased at comparable levels as before induction quickly after recovery [123, 124]. Consistent with an activating role of ATX1 at dehydration stress-response genes [68, 125, 126], dehydration-induced transcript levels were diminished in *atx1* plants [127]. However, ATX1 does not seem to have a critical impact on drought stress memory in *Arabidopsis*. Indeed, while being less increased than in wild-type plants, trainable genes still produced increased transcripts in trained, relative to untrained, *atx1* plants and retained high H3K4me3 levels during the watered recovery states. ATX1, ATX2, SDG25 and SET DOMAIN GROUP2/ATX-RELATED3 (SDG2/ATXR3) belong to the same class III of H3K4me3 methyltransferases and are thought to act, partially redundantly, as H3K4 methyltransferase [128–134]. More recently, SDG2 has been found to be essential for the full transcriptional activation of various hormone-responsive genes upon hormone treatment (i.e. including the ABA-dependant *RD29A*) via its H3K4 trimethyltransferase activity [135]. It is, therefore, likely that the other class III HKMT might also contribute to the drought stress memory. In summary, these results suggest that in addition to be a good marker of gene activation when found around promoter and 5' regions of genes, H3K4me3 might also play a role in establishing a transcriptional short-term somatic memory of drought stress when found in gene bodies.

Using a large-scale approach, the distribution of H3K4me2, H3K4me3, H3K9me2 and H3K27me3 was analysed in *Arabidopsis* seedlings, which have been treated with mild salt stress in the seedling stage, resulting in an increased tolerance upon an additional salt stress application [136]. At low resolution in primed seedlings, H3K4me2 and H3K4me3 most commonly consisted of higher peaks of pre-existing enriched histone modification domains, named islands, whereas H3K9me2 produced the least differences. By contrast, majority of differences in H3K27me3 resulted from a higher number of islands with lower genome coverage. At high resolution, changes in H3K27me3 were already detectable a few hours after salt addition, suggesting that demethylation of H3K27me3 operates at a speed that is comparable to that of transcriptional regulation. Interestingly, this effect fades over time; however, it is still clearly visible after a 10-day-growth period in control conditions. In response to a second stress treatment, genes with high responsiveness, such as *HKT1* (i.e. encoding a root-specific Na transporter) and *PIP2E* (i.e. encoding a plasma membrane aquaporin), experienced a decrease of H3K27me3, whereas genes with lower responsiveness, such as *GH3.1* and *GH3.3* (i.e. encoding auxin and JA-amino acid-conjugating enzymes, respectively), experienced an

increase of H3K27me3. Conversely, another group reported that in plants that have experienced several exposures to dehydration stress no significant change in the level of H3K27me3 could be detected on trainable and non-trainable genes, or during transcriptionally active/inactive gene states [137]. However, the high H3K27me3 level present at inactive dehydration stress memory genes did not interfere with the transition to an active transcription and with the accumulation of H3K4me3 [138]. Together, the function of H3K27me3 in genes that dynamically change transcription seems to depend on the type of environmental stimuli.

In contrast to H3K27me3, the higher level of H3K4me3 retained at the trainable gene *RD29B*, when its transcription is low, further supports the idea that H3K4me3 works as a ‘memory’ histone mark of a previously active state [138]. Generally, H3K4me3 and H3K27me3 play antagonistic roles in gene transcription and are therefore mutually repulsive at developmental genes [37, 139]. Seemingly, the presence of both marks, referred as ‘bivalent domains’, was first described in mammalian stem cells and was proposed to represent a pluripotent chromatin state that poises genes for activation upon appropriate developmental cues [140, 141]. Further work is required to determine whether H3K4me3 and H3K27me3 co-exist at certain genes whose expression is rapidly altered in response to environmental stimuli.

Because plant stress research has traditionally focused on single stresses, we separately described priming in response to abiotic or biotic environmental cues. However, in nature, plants are constantly exposed to mild environmental stresses during their lifetime. While testing how different environmental histories can affect the response of the plant to a subsequent biotic stress, Singh et al. [142] reported that *Arabidopsis* plants exposed to a recurrent abiotic stress (i.e. heat, cold or salt) were more resistant to *Pst* than plants grown in a more stable environment. This enhanced resistance was due to the priming of commonly used marker genes of pattern-triggered immunity (PTI; *WRKY53*, *FLG22-INDUCED RECEPTOR KINASE1* (*FRK1*) and *NDR1/HIN1-LIKE10* (*NHL10*)). Indeed, enrichment for epigenetic marks associated with transcriptional activation, such as H3K4me2 and H3K4me3, at PTI-responsive genes was observed after the exposure to recurrent stress, resulting in an enrichment of RNA polymerase II and a primed transcription in response to a subsequent bacterial infection. Collectively, these works on somatic stress memory promote the idea that, in plant, the environmental history can shape/modulate the response to stress, providing a mechanistic link between histone methylation and gene priming.

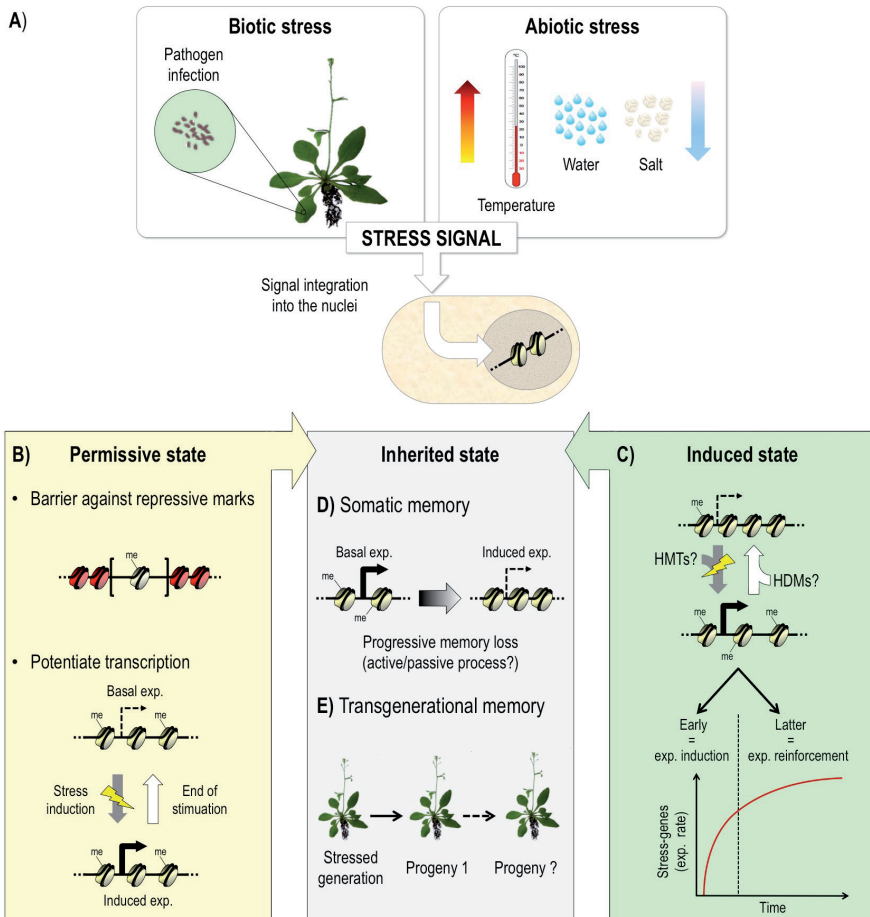
## 5.2. Transgenerational stress memory

The transgenerational stress memory refers to the transmittance of certain environmental responses from one generation to the next, thus providing the offspring of environmentally challenged plants with an adaptive advantage for better fitness (i.e. improve plant stress tolerance and impart developmental flexibility; [143]). Compared with DNA methylation and RNA interference (RNAi), very few studies suggest the involvement of histone methyltransferases and histone methylation changes in this process [144]. In *Arabidopsis*, changes in DNA methylation, histone modifications and gene expression were followed in the progeny of plants exposed to salt stress over one generation [145]. Although the DNA from the progeny of plants exposed to salt stress was globally hypomethylated, the majority of genes and promoters

causing methylation changes were hypermethylated and lowly expressed. In addition, DNA hypermethylation was correlated with an increased level of the repressive mark H3K9me2. Among these hypermethylated genes, a large number was encoding different histone methyltransferases, such as the *Arabidopsis* SU(VAR)3-9 homologues *SUVH2*, *SUVH5*, *SUVH8*, involved in H3K9 methylation, or the PRC2 subunit *CURLY LEAF* (*CLF*), involved in H3K27 methylation. Following this work, the progeny of heat-stressed plants was used to explore epigenetic variations under both normal and stressed conditions, in comparison to the progeny of control plants [146]. Similarly to salt stress, the progeny of plants exposed to heat stress had a global decrease of genomic DNA methylation and a reduced expression of several *SUVH* genes, which correlated with their enrichment in H3K9me2. Together, the hypermethylation of *SUVH* genes in the progeny of stressed plants may represent a protective mechanism against hypermethylation of the entire genome. Interestingly, in both works [145, 146], the transposon expression was elevated in the progeny of stressed plants. Because main targets of the *SUVH* pathway are transposable elements [24], the authors proposed that a decrease in the expression of *SUVH* genes might contribute to transposon activation, which at opportune times can create intragenomic potential upon transposition to facilitate adaptation in response to environmental changes [147]. In summary, these works suggested a role for histone methylation in the inheritance of stress memory; however, whether histone methylation changes are heritable through multiple generations and whether they sustain the acquisition of adaptive traits is still a matter of debate [148]. However, the evidence to date favours the view that stress-induced transgenerational changes in chromatin might increase the survival chances of the plant species, rather than each individual, by broadening the phenotypic plasticity and the genetic variation within the population [149, 150].

## 6. Discussion and perspectives

Recent advances, especially in *Arabidopsis*, have uncovered that chromatin remodelling through histone methylation changes are not only restricted to developmental needs but also an integral part of the very complex cascade of events that lead to abiotic/biotic stress tolerance, resistance and short-/long-term memory. Currently, a preliminary view is emerging, indicating that histone methylation changes, providing specific chromatin configurations, can be classified into several interrelated categories when involved in stress responses (Fig. 1): (i) histone methylation changes that are basally present on stress-related genes to establish a 'permissive' chromatin state that may either limit the spreading of repressive chromatin marks and/or potentiate a rapid transcriptional induction upon need; (ii) histone methylation changes that are transiently induced from an inactive or a permissive chromatin state by stress, to either facilitate the transcriptional initiation and/or reinforce transcription of stress-responding genes, and finally, histone methylation changes that are established in response to a stress; (iii) maintained for a certain time during the lifespan of an individual (i.e. somatic memory) or (iv) transmitted to one or more subsequent generations (i.e. transgenerational memory).



**Figure 1. Hypothetical model depicting the role of histone methylation/demethylation in regulating plant stress responses.** (A) Plants exposed either to biotic or abiotic stresses integrate the signal stress into the cell nuclei, where it affects the chromatin structure through histone methylation changes. According to our knowledge, these histone methylation changes can be classified into several interrelated categories. (B) The permissive state represents a more loosened chromatin state that will either offer a protection against repressive marks (represented with nucleosomes in red) and/or potentiates a rapid transcriptional induction upon stress induction. (C) The induced state represents histone methylation changes that are transiently induced by a stress signal. If methylation changes occur early during the stress-response process, they might participate to the transcriptional induction of stress-responding genes, while if they occur later, they might reinforce the transcription of stress-responding genes. Both permissive and the induced states can be maintained allowing a faster and/or stronger transcriptional induction of stress-responding genes upon a subsequent challenge. The memorized chromatin state can be maintained (D) for a certain time during the lifespan of an individual and referred as the somatic memory, or (E) transmitted to one or more subsequent generations and referred as the transgenerational memory.



Nonetheless, this emerging view is facing many gaps, inaccuracies and divergences, mainly related to numerous difficulties inherent to the study of such a dynamic and acute process. In this respect, plants in nature are usually challenged simultaneously by different kinds of stresses. Responses to these stress combinations are largely controlled by different signalling pathways that can interact in a non-additive manner, producing effects that could not have been predicted from the study of either stress individually [151, 152]. The occurrence of simultaneous biotic and abiotic stresses introduces an added degree of complexity that requires stresses to be imposed simultaneously and to treat each set of environmental conditions as an entirely new stress. For this reason and to clarify the mechanism behind the regulation of stress responses by histone methylation changes, there is a strong necessity to intensify our investigations. For instance, the correlation between histone methylation/demethylation and stress responses remains elusive and clarifications will require in-depth dynamic approaches based on comparative analyses of both epigenomes and transcriptomes during stress responses. In parallel, current knowledge about the corresponding histone-modifying enzymes is still largely missing. This lack of knowledge is pending on the identification of different stress-responsive histone modifiers and will require large-scale screens and genetic analyses for the sensitivity of different histone methyltransferases/demethylases mutants to various stresses, combined or not. Among other factors governing stress-induced chromatin changes, almost nothing is known about the specific reader/effector that will recognize particular histone methylation sites in order to determine their functional and structural outcome. An effort in this direction will most likely benefit the comprehensive understanding of the fundamental mechanisms connecting histone methylation changes with the modulation of transcription of stress-responsive genes, subsequently enabling plant to withstand stress. Higher-resolution chromatin studies are undoubtedly required to reveal the targeted stress-responsive genes and the specific sites of histone methylation/demethylation. Nevertheless, investigation of the direct effects of histone methylation/demethylation in plants is difficult. One reason is that plant genomes harbour high-copy number of histone genes (e.g. the *Arabidopsis* genome comprises 47 genes that encode 33 different core histone proteins; [www.chromdb.org](http://www.chromdb.org)) and the incorporation/modification of such variants can result in the formation of chromatin with particular properties and functions [153–155]. Although ChIP assays have proven valuable in helping to identify histone methylation changes, many antibodies used to detect these changes have been so far unable to distinguish between different variants. New technologies (e.g. generation of mutants with point mutations targeting amino acid in the N-terminal tail of histone using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPER-associated (Cas) system) [156] will need to be explored to unravel histone methylation changes of specific histone variants and their functions. Another challenge is that plants consist of many functionally specialized tissues and cell types, each with its own unique epigenome, transcriptome and proteome. Until now, histone methylation changes induced by stresses were exclusively addressed in entire plant or organs, meaning that the obtained profiles most likely reflect the consensus of multiple tissue- or cell-specific profiles that may differ. New methods allowing the mapping of chromatin features in specific tissue/cell types such as the one described by Wang and Deal [157] will be decisive for determining the cell-/tissue-specific chromatin alterations involved



in a particular stress response. Finally, as plants have finite resources that must be balanced between growth and defence against stresses, often resulting in a growth or yield penalty, histone methylation changes in response to stress should be integrated in a more global developmental view, taking into account the involvement of several histone methyltransferases/demethylases in various processes such as root growth, flowering time, floral organogenesis, gametophyte or embryo formation [33]. Finally, understanding such regulatory network is an essential step to provide both novel paradigms and potential tools for further exploitation towards sustainable agriculture.

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# Small RNAs in Plant Response to Abiotic Stress

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Additional information is available at the end of the chapter

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## Abstract

Small noncoding (nc) RNAs (sRNAs) are the important posttranscriptional regulatory factors in gene regulatory networks. They are involved in many important processes of plant development and stress responses. Increasingly research data reveal that microRNAs (miRNAs), heterochromatic small interfering RNAs (hc-siRNAs), trans-acting small interfering RNAs (ta-siRNAs), natural antisense small interfering RNAs (nat-siRNAs), repeat-associated small interfering RNAs (ra-siRNAs), and the piwi-interacting RNAs (piRNAs) are involved in heat stress, salt stress, cold stress, and drought stress, which are found in metazoans. Some small RNAs are required for plant thermotolerance and salt tolerance. These findings facilitate our investigation of the genetic basis of plant adaptability to various environmental stresses and the genetic manipulation of plant tolerance to many abiotic stresses. This chapter highlights the recent advances in understanding the crucial roles of sRNAs in plant responses to heat, drought, salinity, and cold and proposes the potential technologies and strategies used to identify abiotic-stress-regulated sRNAs in addition to the recent advances and methods for validation and analysis of their target genes.

**Keywords:** Abiotic stress, microRNAs, Plants, Small RNAs, Thermotolerance

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## 1. Introduction

Environmental stresses, such as heat, drought, salinity, nutrient deficiency, and low temperature, are the major natural limiting factors for plant growth and crop productivity and thus are the major causes of crop losses worldwide. In recent years, much progress has been made in unraveling the complex and sophisticated molecular mechanisms by which plants have evolved during periods of environmental stresses, and a great deal of attention has been paid to identifying these stress-responsive proteins and their relevant gene networks. Plant-stress responses depend on the precise expression of the genes and their accurate regulation, which is attained by multiple mechanisms at different levels such as transcriptional, posttranscrip-

tional, and posttranslational regulations. Although studies have been mostly focused on the transcriptional level of regulatory mechanisms so far, recent results lead us to the point that posttranscriptional events also play a very important role in gene expression regulation in major scenarios of a plant life, from developmental processes to stress responses. Small noncoding RNAs (sRNAs) are the important posttranscriptional regulatory factors in gene regulatory networks. They are involved in many important processes of plant development and stress responses.

sRNAs are roughly divided into different categories based on the genomic origins of their precursors: microRNAs (miRNAs), trans-acting small interfering RNAs (ta-siRNAs), and natural antisense small interfering RNAs (nat-siRNAs). These sRNAs are loaded into RNA-induced silencing complexes (RISC) and regulate the expression of their relative target genes negatively by affecting the mRNA levels, chromatin remodeling, and DNA methylation. Understanding of sRNA-guided stress regulatory networks should provide us with new tools and vision for the genetic improvement of plant stress tolerance and eventually developing more stress-resistant plants in future.

This chapter highlights the recent advances in understanding the crucial roles of sRNAs in plant responses to heat, drought, salinity, nutrient deficiency and low-temperature stresses, and proposes potential technologies and strategies used to identify abiotic stress-regulated sRNAs in addition to the recent advances and methods for validation and analysis of their target genes.

## 2. Small noncoding RNAs

The discovery of RNA interference (RNAi) in the late 1990s has been a tornado for the past decade in terms of surprising geneticists for it changed the earlier understanding of the RNA field and the complexity of posttranscriptional control and epigenetic regulation caused by small RNAs. In 1995, Guo and Kemphues used antisense RNA sequence to block the *par-1* mRNA in *Caenorhabditis elegans* when they figured out *par-1* mRNA is repressed by *par-1* mRNA itself [1].

Posttranscriptional gene silencing (PTGS) by RNAs was first reported in plants in 1996. The *lin-4* gene, which is known to be essential for the timing of larval development in *C. elegans*, is controlled by a short RNA, which is not translated to any proteins but has a partially complementary sequence to the 3' region of *lin-4* transcript that inhibited the translation of *lin-4* to protein [2]. Hence, this discovery even changed the concept of PTGS mentioned in high school biology textbooks as it questioned the central dogma proposed by Francis Crick in 1956, which stated that RNAs carry the biological information encoded in DNA molecules and they subsequently provide the code for translation into proteins [3-4]. This discovery called so much attention that was introduced as the breakthrough of the year when it was published in the journal *Science* in 2002 by Couzin and changed the basic concepts about the gene expression and RNA functionality [5]. This was in agreement with the results published by three independent labs that discovered miRNAs in the model plant *Arabidopsis thaliana* [6-8]. They reported many miRNAs in plants, most of which have a very conserved sequence among



different species. Small RNAs could be much more than what was thought. They can influence almost all the functions in a cell by targeting the transcription factors and key genes.

One of the main reasons that small RNAs called so much attention was that soon after their discovery, the target genes for these small RNAs were reported to be crucial in leaf or flower development, which was consistent with the previous reports [9-13]. They have been found to have an impact on almost all the biological processes in eukaryotic cells as they have a wide range of target genes, which are corresponded to some of the previously identified regulatory genes and transcription factors that proved to play key roles such as in controlling cellular metabolism, growth and differentiation, phase transition timing and leaf patterning, and defense mechanisms against biotic and abiotic stresses in case of plants. These 18–25-nucleotide (nt) RNAs are categorized into many different classes based on their size, their biogenesis pathway, and their mode of action.

sRNAs are short nucleic acid sequences that give rise to the assembly of protein–RNA complexes, which later are able to repress the expression of their identified target genes by sequence-specific base pairing. This silencing of the target sequence can occur through several ways by (1) reducing their rates of transcription, (2) reducing the stability of their mRNAs in the cell, or (3) reducing the translation of their mRNAs into protein.

Although much of the work on ncRNAs field has been focused on small RNAs of under 40 nucleotides long, there are larger ncRNAs called mRNA-like ncRNAs (or mlncRNAs) that have received much less attention and have been reported to play some roles in some of the plant functions such as phosphate starvation response and nodulation. The article by Rymarquis et al. explains about them [14].

The generation of sRNAs involves a set of evolutionary conserved proteins, such as Dicer (DCR) or Dicer-like (DCL), Argonaute (AGO), and RNA-dependent RNA polymerase (RDR), which all together form the RNA silencing machinery in plants. The DCLs have been the most studied enzyme so far, which, in *Arabidopsis thaliana*, are classified into four groups: DCL1 acts during miRNA metabolism, DCL2 is responsible for the viral resistance, DCL3 triggers the transcriptional silencing, and DCL4 cooperates in posttranscriptional silencing and ta-siRNA metabolism (dissecting *Arabidopsis thaliana* dicer function in small RNA processing, gene silencing, DNA methylation patterning, and nature genetics). Plant genomes encode only one of the three known classes of AGO proteins, namely AGO1, which is involved in both miRNA and sRNA biogenesis.

The sRNAs are categorized into different classes based on their size, their biogenesis pathway, and their mode of action to at least six groups, including microRNAs (miRNAs), heterochromatic small interfering RNAs (hc-siRNAs), trans-acting small interfering RNAs (ta-siRNAs), natural antisense small interfering RNAs (nat-siRNAs), repeat-associated small interfering RNAs (ra-siRNAs), and the piwi-interacting RNAs (piRNAs), which are found in metazoans.

## 2.1. miRNAs

Typically, miRNAs are derived from single-stranded RNA precursors that are transcribed by RNA polymerase II from MiRNA genes called primary microRNA transcript (pri-miRNA),

which are capable of forming a self-complementary fold-back structure named hairpin or stem loop in which the mature miRNA could reside on the 3' or 5' end (Table 1). This imperfect double-stranded structure is further recognized and processed by DCL1 in association with other protein factors [15]. This gives birth to the miRNA/miRNA\* duplex which based on the thermodynamic features will have a different fate but usually the pre-miRNA strand is loaded onto an AGO1-containing, RNA-induced silencing complex (RISC) and the miRNA\* strand lives for a short time in the cell. Mature microRNAs (miRNAs), which are the so-called hairpin-derived RNAs, are 20–24 nt long and single stranded while miRNA genes are 70–300 nt long. Mature miRNAs help the target recognition and cleavage in cooperation with AGO1 and miRISC. The first cleavage by DCL1 generates a stem-loop intermediate, called the precursor miRNA (pre-miRNA), and the second cleavage by DCL1 releases the miRNA duplex, one strand of which is known as mature miRNA and the other strand is known as miRNA\* (miRNA star).

Class	Full Name	Originating Loci	Function	Biogenesis
miRNA	microRNA	MIRNA genes	Repress target gene expression through mRNA cleavage and translational repression	The fold-back structures of long ssRNA transcripts are cleaved by Dicers
siRNA	short-interfering RNA	Repeats, transposons, and retroelements (endogenous). Transgenes and viral RNAs (exogenous)	Silence repeats and transposons through RNA-dependent DNA methylation and chromatin modification	RDR-generated dsRNAs are cleaved by Dicers
ta-siRNA	trans-acting siRNA	TAS loci	Repress target gene expression through mRNA cleavage	TAS transcripts are cleaved by miRNAs, transcribed by RDR into dsRNA, and then processed by Dicers
nat-siRNA	natural antisense transcript-derived siRNA	Loci producing pairs of sense-antisense transcripts	Stressed-induced nat-siRNA to repress target gene expression through mRNA cleavage	The dsRNA derived from overlapping transcripts is cleaved by Dicers
piRNA	piwi-interacting RNA	Repeats, transposons, and retroelements	Germ-line-specific piRNA to suppress repeats and transposons in flies and mammals	ssRNA derived from transposons is cleaved by PIWI protein

**Table 1.** Small RNAs involved in plant response to abiotic stresses

Mammals use only one class of RNase III enzyme, Dicer, to generate both miRNAs and siRNAs. In plants, there are a variety of specialized DCL endonucleases, which are classified into 10 categories. DCL1 is involved in miRNA biogenesis pathway while other DCLs

participate in various aspects of sRNA-mediated generation or gene silencing pathway (Figure 1) [16].

All the information about the reported miRNAs and their sequences and annotations are stored in a database called miRBase ([www.mirbase.org](http://www.mirbase.org)), which is updated on a regular basis with the new published data in the literature [17]. So far, there are 205 precursors and 384 mature miRNAs reported in the model plant *Arabidopsis thaliana*, which is about 1–2% of its genome. Theoretically, the perfect base pairing between miRNAs and complementary target mRNAs helps the process of finding target genes for each miRNA. By computational methods, there are a plenty of databases that are able to predict the potential target genes for each newly found miRNA.

## 2.2. siRNAs

siRNAs were first identified in 1999 in plants [18], and later there were many reports about diverse sets of endogenous siRNAs in plants as well as in animals (Table 1) [6] [8][19][20-22]. Most of the plant siRNAs are around 24 nt in length that are excised from the long double-stranded RNA duplexes or transcripts generated from inverted repeat regions [23-24]. The sources of these double-stranded sequences that eventually trigger biogenesis of siRNAs could be endogenous or exogenous. Endogenous plant siRNAs can be classified into several categories, including miRNA-induced trans-acting siRNAs (tasiRNAs), natural antisense siRNAs (nat-siRNAs), cis-acting siRNAs (casiRNAs), heterochromatic siRNAs, and many other unclassified small RNAs [25]. In plants, ta-siRNAs are generated from the genomic loci named TAS genes, which are transcribed by RNA-pol II. The generation of ta-siRNAs is triggered by an miRNA, which cleaves a nonprotein-coding transcript of a tasiRNA gene [26-29]. In plants, there are eight TAS loci reported so far, which belong to four families (TAS1–4). TAS1 and TAS2 families are cleaved by miR173 with the association of AGO1. TAS3 family transcripts are cleaved by the guidance of miR390 and AGO7 and usually target the auxin response factor (ARF) transcripts. TAS4 transcript is cleaved by miR828 guided together with AGO1 and they usually target myeloblastosis (MYB) transcription factors [30]. These cleaved RNAs are then processed by the suppressor of gene silencing 3 (SGS3) and copied into double-stranded RNAs by RNA-dependent RNA polymerase 6 (RDR6). DCL4 cleaves them in multiple rounds so that it finally gives rise to the 21-nt ta-siRNAs. ta-siRNAs are loaded onto AGO1 complex to degrade the target mRNAs [31].

The other class of siRNAs called nat-siRNAs are separated into two groups: cis-nat-siRNAs that are generated from two RNAs, which were transcribed from the same loci but opposite strands, and trans-nat-siRNAs, which were transcripts from different loci [32]. RDR6 and DCL2 are involved in generating 24-nt nat-siRNAs, and RDR6 and DCL1 are involved in generating 21-nt nat-siRNAs. trans-nat-siRNAs are transcripts from different loci but processed by the same proteins (RDR6 and DCL2). ta-siRNAs cleave the target mRNAs by being partially or fully complementary with them.

Heterochromatic siRNAs mostly originate from transposable elements or repeats and their mode of action is slightly different from miRNAs and ta-siRNAs, as they modulate the histone

modification at their homologous regions in the genome and inhibit the gene expression at the transcriptional level.

### 2.2.1. *nat-siRNAs (natural antisense siRNAs)*

Natural antisense transcripts (NATs) are small RNA molecules, which are endogenous and show partial or entire complementarity to other transcripts (Table 1). *cis*-NATs are categorized in the *nat*-siRNAs group and are transcribed from the same genomic loci but in the opposite strand of DNA as their sense transcripts. This class of NATs is very common in eukaryotes (17–30% of the genes encode complementary *cis*-NATs in animals and plants) [33–37]. In animals, NATs are involved in alternative splicing, DNA methylation, RNA editing, and genomic imprinting [38–41]. In plants, several *cis*-NATs are involved in gene regulatory mechanisms [42–43]. There are already some reports about the identified *cis*-NATs in *Arabidopsis* and rice on the genome-wide scale [44–46].

### 2.2.2. *ta-siRNA (trans-acting short interfering RNAs)*

*ta*-siRNAs are 21 nt in length and are reported to be found only in plants so far (Table 1). *ta*-siRNAs originate from a noncoding RNA precursor, which is initially targeted to be cleaved by an miRNA molecule. RNA-dependent RNA polymerase converts the cleaved products into double-stranded RNA molecules, which are later cleaved again into 21-nt *ta*-siRNAs. Hence, the formation of these RNAs is determined by the presence of both miRNA (Dicer-Like1, Argonaute1, HYPONASTIC LEAVES1, and HUA ENHANCER 1) and siRNA (RNA-dependent RNA polymerase 6 and DCL4) biosynthesis pathways components. *ta*-siRNAs can guide cleavage of target mRNAs and regulate gene expression at the posttranscriptional level like plant miRNAs.

## 2.3. Small RNAs in abiotic stress

Abiotic stress is known to be one of the attention-calling factors globally, which causes a considerable yield loss each year. Hence, much effort has been made in understanding the complex stress-response mechanisms, especially in the identification of stress-responsive protein-coding genes. But, in recent years, after the discovery of small noncoding RNAs, they have been found to be involved in plant stress responses and indeed very functional players in these pathways. These small RNAs regulate the gene expression in different levels and hence are entangled within all the vital pathways in the plant development, metabolism, and stress response.

As sessile organisms, plants have evolved their specific adaptation and acclimation mechanisms in order to survive during the hard spell. To do the morphological and physiological adaptations to abiotic stresses, the plant needs to manage the complicated rearrangement of gene expression networks, which are controlled at transcriptional and post-transcriptional levels. The concern about future food shortages makes it imperative to better understand the genetic control of stress tolerance networks and pathways and to use this knowledge to increase the total tolerance of important crop species. As the first step, we have to understand

the complex responses of the plants to stress, from changes in molecular level to physiological level.

With the help of high-throughput gene expression analysis, there have been many reports about the modulated genes and different small RNAs under abiotic stresses. The difference in the expression level of these genes could indicate that it might be responsive to stress condition and, as a result, it can help the plant to survive the hard condition. Many studies have been published in this regard in various plant species, some of which having an economic importance, like rice, wheat, legumes, barley, sugarcane, potato, and tomato as well as many other species. Some of the genes are induced after facing the stress conditions in these studies while some are downregulated, which is connected with the roles these genes play during the stress condition. Also, the respective miRNAs or other sRNAs that target these mRNAs show a different expression pattern during the stress condition. sRNAs, which are accumulated by stress, might downregulate their target genes and act as a negative regulator of stress tolerance; for instance, the genes involved in cell expansion and division should be downregulated as the plant needs to save energy in order to pass through the hard environmental condition. On the other hand, reduction of sRNA level might lead to upregulation of their target genes, mRNAs, which positively regulates the stress tolerance.

The molecular basis of plant tolerance to abiotic stresses and stress regulation of small RNAs has been studied using different methods to observe the altered expression of these molecules and their related target genes; for instance, the sequence analysis of small RNA libraries before and after the stress condition, microarray data analysis, mutagenesis, and RNAi. Their reports have identified numerous genes and sRNAs that are induced by applying different stress conditions, which is the material for the next step: making transgenic plants and check if these overexpressed transgenics could exhibit an improvement in stress tolerance. But the fact is that even though some of the genes and small RNAs show altered expression under stress, they do not play any role to make the plant more tolerant to the stress. And the reason is largely because of the complex genetic interactions underlying the plant tolerance toward stress, which are still to be understood.

From transcriptomic studies, we know that the stress conditions such as heat, drought, cold, and salt evoke the expression of an overlapping set of genes, suggesting that their signaling transduction pathways share common control points. Most of the genes, which are detected to be responsive to abiotic stresses, are usually the genes that regulate plant development and reproduction (as the plant faces the urge to save more energy for producing viable seeds rather than a high biomass), also senescence-related genes (as to recycle the nutrients from the old leaves to younger leaves and reproductive parts and wasting less water and energy for them), as well as the genes that are involved in the abscisic acid (ABA) pathway, which play a crucial role in plant growth and development pathway and redox pathway.

RNA interference technology is one of the potential reverse genetics tools for understanding the functional significance of these genes and their respective regulatory sRNAs. The information about stress-induced genes and sRNAs including the sequence and annotation could be found in the genome databases like National Centre for Biotechnology Information (NCBI) or stress complementary DNA (cDNA) databases. In addition to these stress-induced genes,

the regulatory elements for these genes are also altered during stress condition including small regulatory RNAs. A number of these sRNAs, which are induced in different plant species and under different stress conditions, can be found in some recent review papers [47]. A vast amount of data has been published about the expression profiling of different sRNAs in various stress conditions. Although these expression-profiling experiments can provide us with some clues about the involvement of these sRNAs in gene regulation under those specific circumstances, to find the relevance of each of these sRNAs in imparting stress tolerance in plants can only be studied by functional genomic approaches like gene overexpression or downregulation. RNA interference technology using constructs transcribing self-complementary hairpin RNA is one of the reverse genetics approaches to downregulate genes in plants.

Another powerful technique to learn about gene functions in a developmental or physiological context in plants is by mutagenesis and to isolate the corresponding mutants with altered phenotypes. Various mutagenic agents, including chemical and biological, have been widely used in this regard, each of them with its own advantages and inconveniences.

For *Arabidopsis thaliana*, the genome sequence is publicly available; hence, relying on reverse genetics to understand the relevant roles of genes is currently a common practice. There are specific screening methods used in order to measure the effect of each stress on the overall plant physiology after the treatment as well as the methods used for inducing the specific stress condition in the plant, which have been studied and reviewed many times.

## 2.4. Drought stress

Drought stress is known as the most significant stress especially with regard to the climate change and global warming. It restricts plant growth and development severely, while tolerant plants are able to survive by several mechanisms such as consuming small amounts of water or keeping their stomata closed at a high rate under drought conditions.

Approximately two-thirds of the potential yield of major crops are lost every year due to the adverse growing environments [48]. A worldwide increase in arid areas, including the Mediterranean basin, has been predicted by the International Plant Protection Convention (IPPC) in 2001 and 2007. Water deficit also leads to salinity stress in many cases, which makes the growth situation even harder for the plants. Therefore, it is regarded as the most important abiotic stress and it is necessary to develop strategies toward sustainable use of water and improve plant-drought resistance [49]. Many genes have already been studied and reported to be involved in the drought-resistance response network in the plants. But in recent years, it has become clear that sRNAs play pivotal roles in stress responses as well in regulating the expression of resistance genes.

MiRNA-expression profiling under drought stress has now been performed in *Arabidopsis*, rice, and *Populus trichocarpa*, and many other plants under drought-stress conditions and some of the miRNAs were shown to be responsive toward this stress in different plants, some of which can be reviewed in the available literature [50-51].

Another group has worked on miRNA expression patterns of drought-resistant *Triticum turgidum* ssp. *Dicoccoides* in response to drought stress, using an miRNA microarray platform

[52]. MiR474, which targets proline dehydrogenase (PDH), was upregulated during drought stress in *Zea mays* [53]. Zhao et al. worked on miR169g and miR393 under drought condition in rice, while miR393 was conversely induced by drought [54]. Many other recent reports used several different methods to study the expression pattern changes of miRNAs in different plants under the stress condition [47].

## 2.5. Salt stress

Crops worldwide are threatened by excessive soil salinity due to the accumulation of salt delivered along with irrigation water and by coastal flooding and the high evapotranspiration rates caused by climate change. About 6% of the total arable land in the world is affected by excess salt [55] and it has been predicted to increase to about 30% of the world's arable land by 2025 and 50% by the year 2050 [56]. Several genes and pathways in plants are affected by salt stress [57]. Hence, the promising approach to address the problem of soil salinity is to increase the understanding of response of plants to salinity-related stress. These genes are mostly involved in signal transduction, activation of ion channels, and growth-factor-regulated modification of plant architecture, and, in particular, root morphology.

Besides the genes, numerous differentially regulated miRNAs have also been identified in salt-stressed plants. For instance, miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR319, miR393, miR394, miR396, and miR397 were all reported to be overexpressed in response to salt stress in *Arabidopsis*, while the accumulation of miR398 was downregulated [50].

miR169 was also reported to be induced by high salinity stress [46]. The authors found a cis-acting ABA-responsive element (ABRE) in the upstream region of miR169n, which suggested that miR169n might be regulated by ABA. Another group used microarray experiments as a method to explore the miRNA profile of maize in different lines (salt-tolerant and salt-sensitive); finally, it was reported that the expression levels of miR156, miR164, miR167, and miR396 family members were downregulated considerably, while it was increased in miR162, miR168, miR395, and miR474 families after salt-shock in root tissue [58].

## 2.6. Cold stress

Some plants increase their tolerance to cold in order to deal with the low temperatures. This phenomenon is known as cold acclimation. In recent years, many cold-regulated genes have been identified in plants under cold stress. The C-repeat binding factor (CBF) cold-responsive pathway was considered as the most known cold tolerance pathway in plants [59]. There are three CBF/DREB1 family members, including CBF1, CBF2, and CBF3 (DREB1b, DREB1c, and DREB1a, respectively), encoding the DNA-binding proteins of Apetala2/ethylene responsive factor (AP2/ERF) family [60]. Also, the expression of many miRNAs in cold stress has been examined in different plants including *Arabidopsis thaliana*. Several miRNAs belonging to different families were reported to be upregulated under cold-stress condition in *Arabidopsis thaliana* (miR165/166, miR393, miR396, and miR408), while some other miRNAs (miR156/157, miR159/319, miR164, miR394, and miR398) were shown to be either transient or mildly regulated under cold-stress treatment condition [19][50]. In another report, the expression



levels of miR168 and miR477 family members were increased after the cold-stress treatment, while miR156, miR475, and miR476 members were downregulated in *Populus* plants [55][19].

## 2.7. Heat stress

The average temperature of our planet is rising year by year because of the climate change. As a result, changes in the patterns of rainfall, droughts, and submergence stress are induced to the natural environments. Heat stress even alters the distribution and productivity of important crops negatively throughout the earth. A temperature rise of  $-5^{\circ}\text{C}$  above the plant's optimum temperature is considered as a heat stress. It disrupts normal functions of cellular processes, may lead to delay in plant growth and development, and it might even result in death of the plant, but, usually, high temperatures result in water deficiency, which eventually leads to increase in salt concentration. Recent studies indicate that the projected global warming in the upcoming years will negatively affect the yield of important crops; hence, the necessity of focusing on gene networks and their regulatory components becomes obvious.

A major component with regard to responding to heat stress is the induction of heat shock proteins (HSPs), which get activated by heat shock transcription factors (HSFs). There are five classes of HSPs based on their molecular weights: HSP100, HSP90, HSP70, HSP60, and small heat shock proteins (sHSPs, 15–30 kDa). On the other hand, HSFs recognize heat stress elements on the promoter of heat stress-responsive genes (HSE: 5'-GAAnnTTC-3'). Plant HSFs are categorized into three classes based on their oligomerization domains (A, B, and C) [61].

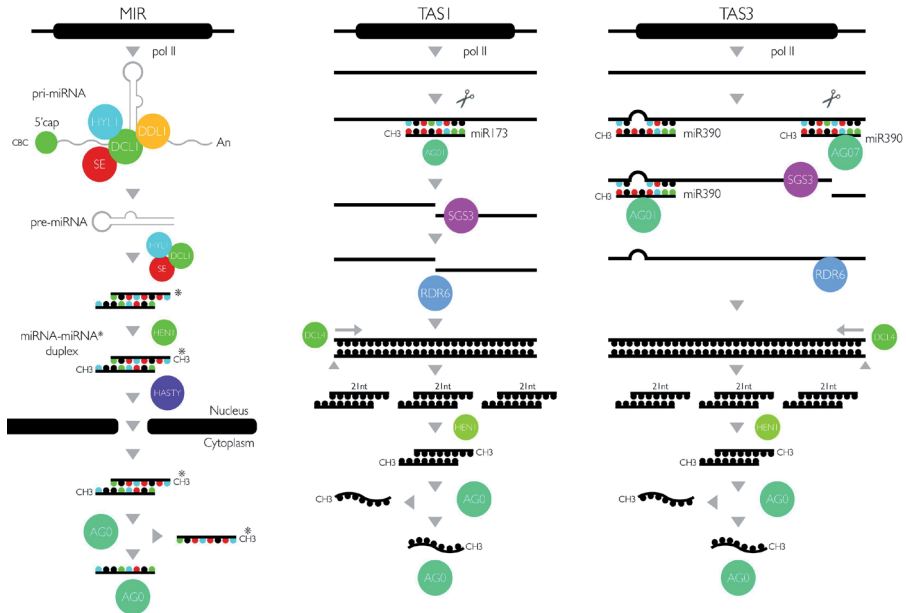
However, the more upstream regulators of HSFs remain to be identified. Guan et al. have reported that miR398 is rapidly induced by being subjected to heat stress while its target genes (CSD1, CDS2, and CCS) are downregulated. They further reported that the expression levels of HSF and HSP genes in *csd1*-, *csd2*-, and *ccs*-mutant plants are increased under heat stress, and *csd1*, *csd2*, and *ccs* plants are more tolerant to heat stress than wild-type plants. They identified two HSFs, which act upstream of miR398, suggesting that this pathway is an essential regulatory loop for plant thermotolerance [62].

Based on deep sequencing experiments, Wang et al. suggest that there is a new class of small RNAs that originate from the chloroplast genome, which are responsive to heat stress [63]. They performed RNA sequencing (RNA-seq) and found 1031 cis-NATs in *Brassica rapa* based on the homology with *Arabidopsis* and 303 conserved cis-NATs, which correspond to the ones in *Arabidopsis* [64]. TAS1 (trans-acting siRNA precursor 1) targets, derived from small interfering RNAs named heat-induced TAS1 target1 (*HTT1*) and *HTT2*, are involved in thermotolerance [65]. *HTT1* and *HTT2* genes were highly upregulated in *Arabidopsis thaliana* seedlings in response to heat shock based on their microarray analysis. TAS1a has a trans-acting small interfering RNA, which targets the *HTT* genes. Overexpression of TAS1a accelerated the expression of TAS1-siRNAs and decreased the expression levels of *HTT* genes that eventually led to weaker thermotolerance. Conversely, stronger expression of *HTT1* and *HTT2* genes upregulated various *Hsf* genes, helping the plants to achieve a stronger thermotolerance. In *HsfA1a*-overexpression transgenic plants, which present a higher tolerance to heat stress, the *HTT* genes were upregulated. In the meantime, *HsfA1a* was shown to bind to the *HTT1* and



*HTT2* promoter regions and activate them directly. Finally, they proposed that *HTT1* interacts with Hsp70-14 and Hsp40, nuclear factor Y, and subunit C2 complex.

Wheat miRNAs showed differential expression in response to heat stress; by using Solexa high-throughput sequencing, Xin et al. cloned the small RNAs from wheat leaves treated by heat-stress gene [66]. Stief et al. also reported that miR156 is responsible for heat stress memory in *Arabidopsis* [67].



**Figure 1.** Biogenesis of miRNAs and ta-siRNAs

### 3. Perspective

Physiological responses to stress are controlled by expression of a large number of genes, many of which are regulated by microRNAs. At the molecular level, identification of stress-responsive genes is an initial step toward understanding plant stress response as pyramiding of different genes in the same plant is an option for achieving better stress tolerance. Although finding genes and sRNAs, which show induction by stress, is an important step toward stress tolerance improvement, most of the studies in which they use transgenics only show the importance of the introduced transgene and not the overall metabolic effects that the trans-host gets exposed to. On the other hand, the new stress-tolerant transgenic lines should have

no or few undesired phenotypic changes plus a minimal yield penalty. In stress-tolerant transgenics, which are introduced so far, a constitutive promoter has been used for expressing the transgene in most of the cases. These transgenes must be utilized to overcome the problem of yield penalty and growth retardation in these experiments. Admittedly, most reports published on stress-tolerant transgenic plants are based on the limited characterization of the stress condition as well as the tolerant phenotypes. Adequate assays for phenotyping of the stress-tolerance trait must be undertaken under natural stress conditions. Overall, there is a lack of uniformity in the stress induction regimes applied by various research groups, which makes the comparisons of the responses among different reports difficult and this fact must be taken into consideration.

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# **The Transcriptional Modulation of Inositols and Raffinose Family Oligosaccharides Pathways in Plants — An (A)Biotic Stress Perspective**

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Additional information is available at the end of the chapter

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## **Abstract**

Among the multifunctional molecules that participate in processes of plant tolerance/resistance to stresses, inositol (Ins) and its derivatives (phosphorylated, methylated, oxygenated, and Raffinose Family Oligosaccharides) have attracted the attention of researchers. These compounds represent versatile and dynamic signaling molecules and osmolytes in all eukaryotes. Due to the impacts related to Ins and its derivatives in a plant cell, assays have been conducted to understand how these biomolecules affect plant physiology. Thus, overexpression or knockout of Ins-related genes has been shown as interesting strategies for generating more efficient plants capable of growing under stress conditions. In this chapter, studies using molecular tools are presented, and the impacts of their results are discussed based on the plant stress tolerance/resistance. Furthermore, an informative panel is provided with transcriptional modulation of genes related to Ins and its derivatives expressed in plants under stress. There is a gap involving about two dozen enzymes associated with the synthesis of Ins-related compounds that have not been adequately studied, and they represent an area of high biotechnological potential.

**Keywords:** Transgeny, tolerance, resistance, biotechnology

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## **1. Introduction**

To survive and integrate in the niche in which they germinate, plants constantly regulate their internal environment to external fluctuations encompassing soil, climate, and biological interactions. Thus, along its evolutionary processes, plants were selected through the need of

molecular mechanisms for physiological adjustments to inadequate conditions for development, resulting from adverse conditions. In this way, plants have a diverse and active cellular machinery at different stratified levels, covering perception, signaling, transcriptional control of key metabolic pathways and synthesis of molecules responsive to stresses [1].

Among the molecules functioning in more than one of the aforementioned levels, inositol (Ins;  $C_6H_{12}O_6$ ) is a biomolecule of great interest. It is a cyclic carbohydrate (polyalcohol) that anchors in each of the six carbons forming the ring, a hydroxyl group. Along with their derivatives, Ins has multiple effects on plant metabolism. They act from the production of secondary messengers to the synthesis of osmolytes and antioxidants (more details in the reviews of [2, 3]). Phosphorylated Ins-derivatives [(poly)phosphoinositides and inositol (poly)phosphates] are versatile and dynamic signaling molecules in all eukaryotes, particularly in plants [4]. These two classes of compounds [highlighted in red and orange respectively, in Figure 1] are interdependent. While (poly)phosphoinositides are used in the synthesis of inositol (poly)phosphates through the action of phospholipases; the breaking of inositol (poly)phosphates produces inositol, which is a substrate for the synthesis of (poly)phosphoinositides. Moreover, according to Ins metabolism, shown in Figure 1, another branch realizes the synthesis of methylated derivatives (highlighted in green). These compounds act as important osmoregulators during periods of unfavorable conditions [5]. Additionally, oxygenated Ins-derivatives are observed (highlighted in yellow in Figure 1), which are involved in increasing plant tolerance to stresses by decreasing oxidative damage [6]. Still associated with Ins is the metabolism of the Raffinose Family Oligosaccharides (RFOs) [7]. In this biosynthetic pathway, the galactinol synthase (GolS; EC 2.4.1.123) uses myo-inositol and UDP-galactose to produce galactinol, which serve as galactose donors for subsequent synthesis of RFO members ([8, 9]; highlighted in red in Figure 2). Recent reports indicate that RFOs may assist in the reactive oxygen species (ROS) cleaning process. In periods of stress, ROS accumulation favors the physiological imbalance of plants [10].

Due to the impacts related to the Ins and its derivatives in a plant cell, assays have been conducted to understand how these biomolecules affect the physiology of plants. Thus, overexpression or knockout of genes present in these pathways has been shown as interesting strategy for generating more efficient plants capable of growing under abiotic stress conditions. In this chapter, studies using molecular genetic tools will be presented, which affect the above-mentioned metabolic pathways and the studied organisms.

## 2. The use of Ins and phosphorylated Ins-derivatives in plant cells under stress

Although there are several articles addressing the involvement of Ins and its derivatives in plant stress responses, so far no report has described the overall transcriptional orchestration of these components covering the metabolic pathways related to them. Information is available only in particular assays covering few genes and their expression modulations, some individual gene knockout analyses or a specific GMO (genetically modified organism) assay. In most

cases, Ins and its phosphorylated derivatives have positive impact in plant tolerance/resistance to several stresses, promoting a biotechnological interest in these compounds.

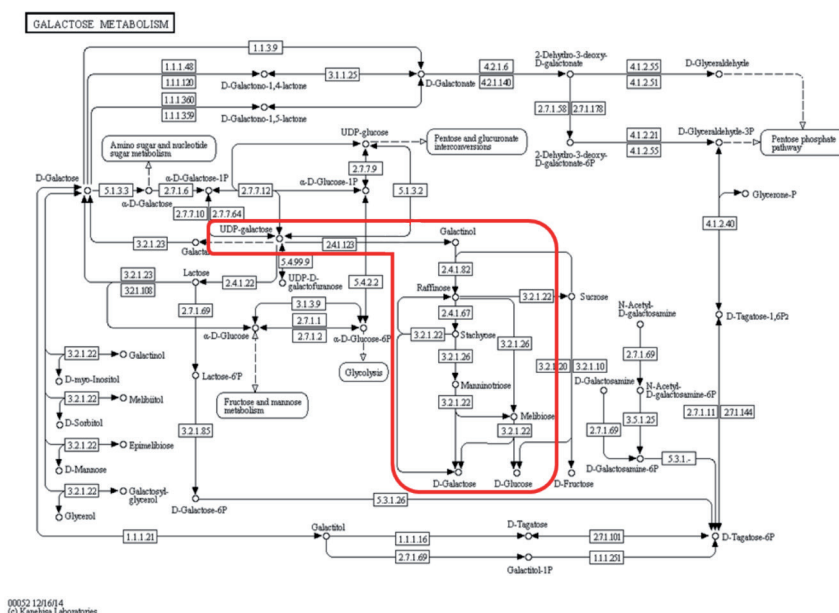
Among the Ins-derivatives, those that are phosphorylated [(poly)phosphoinositides (highlighted in red in Figure 1) and inositol (poly)phosphates (highlighted in orange)] are the most discussed in the literature. This fact reflects the importance of these compounds in plant physiology in signaling activities. An example is a work developed by Hunt et al. [11] with transgenic tobacco plants (*Nicotiana tabacum* cv. Wisconsin 38) expressing reduced levels of a gene encoding phospholipase C (PI-PLC; EC 3.1.4.11). This enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to InsP3 [inositol (1,4,5)-trisphosphate; (Figure 1)], affecting from this point on the rest of the pathway. The obtained transgenic plants showed a partial inhibition of stomatal opening due to the action of ABA (abscisic acid phytohormone). A possible role of PI-PLC enzyme and InsP3 in ABA-dependent signaling pathway was also reported, thus suggesting that a complete response to ABA phytohormone in guard cells requires PI-PLC. However, other calcium-mobilizing pathways could also help in ABA guard cell signaling. The stomatal movement is a critical process for the plant under drought conditions by enabling better use of its water supply.

Mills et al. [12] provide more details on this ABA-mediated stomatal regulation in transgenic plants obtained by Hunt et al. [11]. A three-day assay under drought and in dark-adapted conditions, to reopen the stomata in response to light, was carried out. The results showed that transgenic plants with reduced PI-PLC as compared to control plants (with the empty vector without the transgene insert) have a greater increase in stomatal conductance. Thus, there is a strengthening of the role of inhibition due to PI-PLC in ABA-mediated stomatal opening. Further analysis indicated tobacco PI-PLC acting on the inhibition of stomatal opening by ABA, but not in promoting ABA-induced stomatal closure.

There are also reports of the involvement of Ins-derivatives in ABA-independent mechanisms during periods of drought. Perera et al. [13] obtained *A. thaliana* plants transformed with human type I gene for inositol polyphosphate 5-phosphatase (InsP5-ptase; EC 3.1.3.56; Figure 1). This enzyme hydrolysis InsP3, which is an essential element of the signal transduction pathway in general response to stresses. Looking at the impact on plant response to drought, the authors observed that transgenic plants showed increased stress tolerance after 12 days of watering suspension. After this period of stress, wild and controls plants (with the empty vector without the transgene insert) became brown and dry, while InsP5-ptase transgenic lines remained green and turgid. Furthermore, transgenic plants under drought conditions showed reduced levels of ABA compared with wild plants in the same condition as well as no induction of several genes regulated by the phytohormone. The analyses of stomatal responses in transgenic plants observed that guard cells are less responsive to the inhibition of opening stomata promoted by ABA. Nevertheless, there is an increase in sensitivity to the closing of the stomata, induced by the phytohormone. The transgenic plants showed irregular behavior in coordinated processes via ABA. Despite this, these plants showed a compensatory overexpression of an ABA-independent pathway involving the transcription factor (TF) DREB2A (dehydration-responsive element-binding protein 2A) and a subset of genes regulated by this TF. In this way, the drought tolerance of Ins5-ptase plants was mediated in part via DREB2A-

dependent and that constitutive dampening of InsP3 signal revealed unforeseen interconnections between signaling pathways.

The involvement of InsP3 in other stress tolerance processes, beyond drought, has also been demonstrated. Alimohammadi et al. [15] obtained transgenic tomato plants (*Lycopersicon esculentum* cv. MicroTom,) overexpressing a human type I InsP5-ptase gene. These plants presented a decreased level of InsP3 and supported a continuous exposure to light longer than wild plants. Prolonged exposure to light causes oxidative stress in plant cells and can result in irreversible damage. However, the molecular mechanism involved in this tolerance process was not reported, but these transgenic lines were characterized in more detail by Alimoham-



**Figure 2.** The Galactose metabolism from the KEGG Pathway database. Highlighted in red are the enzymatic reactions associated with the metabolism of Raffinose Family Oligosaccharides.

madi et al. [16]. These authors observed that under stress conditions, the transgenic plants maintained high chlorophyll content and accumulated low levels of hydrogen peroxide. This fact was attributed to the induction of genes related to multiple antioxidants [LeAPX1 (*L. esculentum* ascorbate peroxidase 1), SICAT2, LeSOD (*L. esculentum* superoxide dismutase)] during continuous exposure to light. Other effects included overexpression of the LePHYB photoreceptor (*L. esculentum* phytochrome B) and a key enzyme [LeCHS1 (*L. esculentum* chalcone synthase)] in the biosynthesis pathway of flavonoids, which are plant nonenzymatic antioxidants. There was also an overexpression of the SIMYB12 transcription factor, leading to an increase in flavonoids in tomato plants by up-regulation of the LeCHS1 expression. A relationship was established between change in phosphoinositol signaling pathway and increases tolerance to continuous exposure to light, through the activation of ROS-scavenging enzymes, and up-regulation of molecular activators of non-enzymatic antioxidants.

The biotechnological potential through the manipulation of compounds shown in Ins-related metabolic pathways may also be seen in the work of Ahmad et al. [17]. These authors performed a comprehensive analysis of *A. thaliana* genome, using the activation tagging technique in dedifferentiated calli, to identify salt-tolerant mutants (NaCl 150 mM). To this end, plants were modified with pRi35ADen4 binary vector. Such vector contains four copies of the 339 bp long cauliflower mosaic virus (CaMV) 35S enhancer in the construct that induces the expres-

sion of adjacent genes after proper insertion. Of the 18 potential tolerant mutants (150 mM NaCl), a line (stc1; salt-tolerant callus 1) presented the gene for myo-inositol-1-P-synthase-1 [At4g39800; MIPS1; EC 5.5.1.4; Figure 1] with induced expression in callus, with or without salt. This gene expressed 45 and 15 times higher compared to wild-type under the control condition. MIPS catalyzes the first step in the biosynthesis of inositol from glucose-6-P (Figure 1). The referred induction was greater in the mutant line than in the wild type (approximately, 260 times higher), both under stress. The *mips* gene transcription in the wild type reduced dramatically under stress condition. The tolerance analysis revealed that the mutant plants regenerated from calli showed salt tolerance in germination and growth. However, the mechanism involved was not disclosed, but the authors suggested that MIPS protect the calli and the plants from salt stress as osmolytes or by providing a precursor in the regulation of signal transduction pathways.

Kusuda et al. [18] went beyond the study of transformed lines overexpressing enzymes from the Ins-related pathways. They analyzed the differences among wild type and transformants lines in regard to salt tolerance in 3.5 days in medium with concentrations up to 250 mM NaCl. They also sought for differences by mining the metabolomes (the fourth leaf tissue harvested at 0, 6, and 12 h after NaCl stress induction) of the studied plants. To this end, a rice cultivar (*Oryza sativa* cv. Kitaake) was transformed with the construction Act::RINO1. The RINO1 gene encodes an MIPS (EC 5.5.1.4, Figure 1). It has been demonstrated that the constitutive overexpression of rice MIPS when compared to a wild type, results in greater tolerance to salt stress. Furthermore, it leads to a range of metabolic changes, with increased production of various metabolites (such as inositol, raffinose, ascorbate, amino acids). These handle the protection of plants from abiotic stresses. Additionally, activation of basal metabolisms such as glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle has been observed during induction of the Ins metabolism in those plants overexpressing MIPS.

Ins metabolism and phosphorylated Ins-derivatives are also associated with response to biotic stresses in plants. This fact shows the plurality of actions of these compounds. Murphy et al. [19] report evidence in this direction. They obtained transgenic potato [*S. tuberosum* L. (cv. Desiree)] and *A. thaliana* lines, synthesizing low levels of phytic acid (1-D-myo-inositol-P6 or InsP6, Figure 1). The transgenic potato lines were obtained by: (1) constitutive expression of an antisense sequence of the myo-inositol 3-phosphate synthase gene. This enzyme (IPS, EC 5.5.1.4, Figure 1) catalyzes the first step in the InsP6 biosynthesis; (2) plants expressing the *Escherichia coli* polyphosphate kinase (PPK, EC 2.7.4.1; Figure 1). PPK inserts inorganic phosphate into chains of phosphate residues linked by phospho-anhydride bonds, and this decreases the phosphate available to InsP6 biosynthesis. Genetically modified potatoes presenting both (1) and of (2) showed increased susceptibility to avirulent pathogen potato virus Y and the virulent pathogen tobacco mosaic virus (TMV). In relation to *A. thaliana*, the authors obtained three loss-of-function mutants [two (*atips1* and *atips2*) involving the gene for IPS (EC 5.5.4.1, Figure 1); and one (*atipk1*) involving the gene encoding inositol polyphosphate kinase (IPK1; EC 2.7.1.158, Figure 1)]. IPK1 catalyzes the final step in InsP6 and without this enzyme, very little InsP6 is done. The disruption of InsP6 biosynthesis in *A. thaliana* resulted in an increased susceptibility to viruses (tobacco mosaic virus), bacteria [*Pseudomonas syringae* pv tomato (Pst) DC3000 and Pst DC3000 AvrB], and fungus (*Botrytis cinerea*). The

increased susceptibility only occurred for *atips2* and *atipk1* mutants. For *atips1* mutants showing also InsP6 depletion, the resistance to pathogens was not compromised. This suggests either that a particular pool of InsP6 regulates defense against pathogens in *A. thaliana*, or IPS1 and IPS2 are differentially regulated, and one cannot compensate for the other. It was observed with regard to the typical responses of both species that levels of salicylic acid (SA), a key molecular signal for establishing local and systemic acquired resistance, were not diminished. Therefore, SA was not responsible for increased susceptibility to pathogens analyzed. Furthermore, a relationship between the inositol phosphate metabolism and basal resistance to pathogens (fungi, bacteria, and viruses) has been established. This indicates that InsP6 is also required as an essential operation signal for regulation and defense in plants, together with several other well-known defensive signals (NO, cADPR, SA and Ca<sup>2+</sup>).

Recently, Meng et al. [20] found that *A. thaliana* *ips1* loss-of-function mutant (*atips1*; IPS; EC 5.5.4.1, Figure 1) exhibits spontaneous cellular death and increased resistance to oomycete *Hyaloperonospora arabidopsis* pathogen. This result, together with the above, supports the premise that a particular InsP6 pool may regulate defense pathways, since *atips1* mutants did not show increased susceptibility to the variety of testes pathogens (Tobacco mosaic virus; *Pseudomonas syringae* pv. tomato (Pst) DC3000 and Pst. DC3000 AvrB; and *Botrytis cinerea*). Additionally, it was observed that spraying the *atips1* mutant plants with Ins could suppress the formation of spontaneous lesions, indicating that Ins production and not AtIPS1 protein is required to prevent the lesion formation. The authors also found evidence for the role of Ins (or Ins-derivatives) in the regulation of programmed cell death.

Reports presented in Table 1 also show the broad roles of Ins and its phosphorylated derivatives in plant cells. From the wide spectrum of analyzed genes, a range of effects on plants at different levels was observed. These effects have shown associations with hormone signaling pathways, such as ABA [12], influences in photosystems [21, 22], with reactive oxygen species (ROS; [23]), with relative water content, with osmotic adjustment [24], among others (Table 1).

### 3. Methylated Ins-derivatives in plant cell and the biotechnological use to increase stress tolerance

Some plants use Ins as precursor of compatible solutes such as D-ononitol and D-pinitol, which act as osmoprotectants (small molecules that act as osmolytes and help organisms survive in extreme osmotic stress [35]). In halophyte ice plant (*Mesembryanthemum crystallinum*), which is considered highly tolerant to drought, salinity, and cold, Ins is methylated to D-ononitol and subsequently epimerized to D-pinitol [36]. The myo-inositol O-methyl transferase gene (IMT1; EC 2.1.1.40; Figure 1) is transcriptionally induced by osmotic stress, whereas neither transcriptional nor enzyme activities is detectable in ice plants under normal growth conditions [37, 38]. Despite the positive influence of these metabolites in plant physiology under abiotic stress conditions, there are less available data for these compounds compared with phosphorylated Ins-derivatives.



EC+ Number	Gene origin (specie)	Transformant (specie)	Gene Modulation	Analysed Condition	Impact on Tolerance	Authors	Notes
5.5.1.4	<i>Pc</i>	<i>Os and Bj</i>	Overexp.	HS	Raise	[25]	a.
5.5.1.4	<i>Pc</i>	<i>Nt</i>	Overexp.	HS	Raise	[21]	b.
5.5.1.4	<i>As</i>	<i>At</i>	Overexp.	HS	Raise	[22]	c.
3.1.3.25	<i>Ca</i>	<i>At</i>	Overexp.	HS, P, PEG, and HT	Raise	[26]	d.
2.7.1.140; 2.7.1.151	<i>At</i>	<i>Nt</i>	Overexp.	HS and OS	Raise	[27]	e.
2.7.1.140; 2.7.1.151	<i>Th</i>	<i>Bn</i>	Overexp.	HS, D, and OS	Raise	[28]	f.
3.1.4.11	<i>Zm</i>	<i>Zm</i>	Overexp.	D	Raise	[29]	g.
2.7.1.159; 2.7.1.134	<i>Os</i>	<i>Nt</i>	Overexp.	HS	Decrease	[24]	h.
3.1.3.57	<i>At</i>	<i>At</i>	Knockout	HS, F, and D	Decrease	[30]	i.
2.7.1.137	<i>At</i>	<i>At</i>	Knockout	HS	Decrease	[31]	j.
2.7.1.67	<i>At</i>	<i>At</i>	Overexp.	HS and ABA	Raise	[23]	l.
2.7.8.11	<i>Zm</i>	<i>Zm</i>	Overexp.	D	Raise	[32]	m.
3.1.3.8	<i>At</i>	<i>At</i>	Overexp.	HS and OSM	Raise	[33]	n.
2.7.1.149	<i>At</i>	<i>At</i>	Overexp.	HS, D, and ABA	**	[34]	–

†Enzyme Commission; \*According to KEGG Database Pathway; – Not observed; Overexp. (Overexpression); **Legend:** HS (high salinity); P (paraquat); PEG (polyethylene glycol); HT (high temperature); OS (oxidative stress); D (drought); DH (dehydration); ABA (ABA hormone); OSM (osmotic stress); F (freezing). **a.** Albeit to a variable extent, overexpression of this gene confers salt-tolerance to diverse evolutionary organisms (from prokaryotes to eukaryotes), including crop plants; **b.** Transgenic individuals presenting retention of approximately 40–80% of the photosynthetic competence under analyzed stress condition; **c.** Transgenic individuals retained more chlorophyll and carotenoid by protecting the photosystem II; **d.** Improving seed germination and seedling growth in transgenic individuals under stress conditions; **e.** Expression patterns of various stress responsive genes were enhanced, and the activities of antioxidative enzymes were elevated in transgenic plants; **f.** The transcripts of various stress-responsive genes are increased in ThIPK2 transgenic plants under salt stress condition; **g.** The sense transgenic plants had higher relative water content, better osmotic adjustment, increased photosynthesis rates, lower percentage of ion leakage and less lipid membrane peroxidation, higher grain yield than the wild type; **h.** The 1,3,4-trisphosphate 5/6-kinase is a negative regulator of osmotic stress signaling in tobacco; **i.** The genetic evidence indicating that phosphoinositols mediate ABA and stress signal transduction in plants, and their turnover is critical for attenuating ABA and stress signaling; **j.** Salt stress responses, such as increased plasma membrane endocytosis and the intracellular production of ROS, are coordinated by phospholipid-regulated signaling pathways; **l.** AtPI4Kγ3 is activated by DNA demethylation and regulates the ROS accumulation induced by high salt treatment or ABA treatment; **m.** ZmPIS regulates the plant response to drought stress through altering membrane lipid composition and increasing ABA synthesis in maize; **n.** AtPAP15 (3-PHYTASE) may modulate AsA levels by controlling the input of myoinositol into this branch of AsA biosynthesis in *Arabidopsis thaliana*. *At*: *A. thaliana*; *Pc*: *P. coarctata*; *Sa*: *S. alterniflora*; *Ca*: *C. arietinum*; *Th*: *T. halophila*; *Zm*: *Z. mays*; *Os*: *O. sativa*; *Nt*: *N. tabacum*; *Bn*: *B. napus*; *Bj*: *B. juncea*.

**Table 1.** Transgenic and knockout plant assays available in the literature related to (poly)phosphoinositides and inositol (poly)phosphates. Relevant information: EC number of the enzyme coded by the studied gene, plant donor species, the genetically modified organisms (transformants), the modulation of the studied gene, the analyzed stress condition, the impact on plant tolerance and physiology (additional details, please see the legends).

Sheveleva et al. [39] were one of the first to report the biotechnological potential of methylated Ins-derivatives. In their work, the authors superexpressed O-methyltransferase (IMT1; EC



2.1.1.40; Figure 1) of *Mesembryanthemum crystallinum* in tobacco (*Nicotiana tabacum* cv. SRI). The transgenic plant increased its tolerance to abiotic stresses [drought and high salinity (50–250 mM NaCl)] when compared to the wild control line. An accumulation of methylated inositol D-ononitol in amounts of fresh weight exceeding  $35 \mu\text{mol g}^{-1}$  in the transformed lines was observed. Besides, the photosynthetic  $\text{CO}_2$  fixation was less inhibited in those plants during drought and salt stress. Further, transformed plants recovered faster than the wild type after rehydration. In turn, Sengupta et al. [40] observed an increase of the D-pinitol synthesis in a wild-type rice (*Porteresia coarctata*) with halophilic characteristics when subjected to high salt environment (400 mM NaCl). An increment also occurred in both transcriptional and proteomic level of IMT1, not observable in domesticated rice under the same condition. The authors also reported an increase in the expression of L-myo-inositol 1-phosphate synthase (PcMIPS1; EC 5.5.1.4; Figure 1), along with the expression of IMT1. According to the authors, this suggests that the accumulation of D-pinitol would be a mechanism regulated by salt stress.

Recently, Zhu et al. [41] used a similar strategy as the one developed by Sheveleva et al. [39] to express in *A. thaliana*, a gene coding IMT1, from *Mesembryanthemum crystallinum*. The transformed plants showed higher growth compared to the wild control line and increased tolerance to cold stress ( $4^\circ\text{C}$ ). This increment in tolerance was attributed to different factors: (1) the electrolyte leakage content in the transgenic plants was significantly lower than that of the wild-type plants after freezing stress, showing less damage to the membranes of those plants; (2) transgenic plants showed lower MDA content than wild-type plants, not only in normal conditions but also after stress; and (3) a higher proline content presented in transgenic lines than in wild type, after application of stress.

#### 4. Oxygenated Ins-derivatives in plant cell and the plant strategy to tackle stress

So far, the myo-inositol oxygenase (MIOX; EC 1.13.99.1; Figure 1) is the only enzyme known by the oxidation of Ins [42]. Its importance in plants stood out from the statement in the Ins metabolism as a precursor in Ascorbic Acid (AsA) biosynthesis in Arabidopsis. In this sense, Lorence et al. [43] observed the expression of a myo-inositol oxygenase (miox4) increasing the content of AsA in leaves (approximately two- to threefold). By that, they anticipated a potential use of the gene by genetic engineering, enhancing levels of this important antioxidant in plants. Further analysis indicated that D-GlcUA (Figure 1), a derivative from MIOX reaction (EC 1.13.99.1, Figure 1), plays a negligible role for AsA biosynthesis [44]. However, MIOX can control the metabolite level of myo-inositol in plants [44].

Nevertheless, the metabolic consequences of MIOX action are still unclear. In order to contribute with information from gene regulation and catalytic activity of this enzyme, Duan et al. [6] performed a functional characterization in rice (*Oryza sativa*), observing its predominant expression in root, with induced transcription under drought stress (20% PEG6000 solution),  $\text{H}_2\text{O}_2$ , high salt (200 mM NaCl), cold ( $4^\circ\text{C}$ ) and Absciscic Acid (100  $\mu\text{M}$ ). Transgenic rice lines overexpressing an MIOX gene showed a higher survival rate than a wild line, when in contact with 20% PEG6000 solution. In the same way, the authors also reported for the

transgenic lines, transcription levels significantly induced for genes coding enzymes associated with ROS scavenging, suggesting an MIOX activity in reducing oxidative stress.

Moreover, Alford et al. [45] reported MIOX enzymes responding to growing conditions of *A. thaliana* in low energy/nutrient environment. Their data supported the hypothesis that MIOX2 and MIOX4 enzymes would be encoded by multiple genes. Furthermore, on plants growing in low energy conditions/nutrients environments, MIOX2 enzyme would have a significant role in providing Ins to many different tissues, whereas MIOX4 would act in supplemental form at some tissues. An analysis of promoters was presented, considering multiple lines of MIOX2p:GUS and MIOX4p:GUS seedlings growing on: (I) no nutrients (agar); (II) low nutrients (agar + 0.5× MS salts); and (III) optimal nutrients (agar + 0.5× MS salts + 3% glucose) in low light (40  $\mu$ E) during seven days. As a result, only MIOX2p:GUS was expressed abundantly in condition I, whereas in condition II, both MIOX2p:GUS and MIOX4p:GUS showed moderate expression, having been more expressed previously.

## 5. Raffinose Family Oligosaccharides (RFOs) and plant strategy to address stresses

RFOs are a class of compatible solutes coming from Ins metabolism ramifications. As mentioned before, the enzyme GolS (EC 2.4.1.123, highlighted in red in Figure 2) connects the metabolism of these compounds, producing galactinol (highlighted in red in Figure 2), which serves as galactose donor for further synthesis of RFO members [8, 9]. To date, structural genomics data and global transcriptome analysis concerning RFOs are only available for corn [46]. For this crop, the authors have performed a genomic identification of genes associated with raffinose metabolism, together with an expression analysis using data-mining from GEO (<http://www.ncbi.nlm.nih.gov/geo>) and PLEXdb databases (<http://www.plexdb.org>). Additionally some transgenic lines overexpressing specific gene isoforms related to RFO pathway, under particular growth conditions, are available for some species. These studies showed a positive impact in some crops.

Taji et al. [47], for example, analyzed the expression of seven genes encoding GolS in *A. thaliana* under different stresses. From those genes, only three (AtGolS1, 2 and 3) were stress-responsive. AtGolS1 and AtGolS2 were induced by drought and high salt (250 mM NaCl), but not by low temperature (4°C). On the other hand, AtGolS3 was induced by low temperatures, but not high salinity or drought. The AtGolS2 overexpression in *A. thaliana* was associated with an increase of raffinose and galactinol and resulted in a reduction of leaf transpiration with a greater drought tolerance. In turn, Dos Santos et al. [48] analyzing three *Coffea arabica* galactinol synthase isoforms (CaGolS1, CaGolS2, CaGolS3) observed a mainly tissue-specific expression but differentiated regulation depending on the applied stress (drought, heat, and high salinity). This reinforces the observation by Taji et al. [47], indicating that different galactinol sets can be necessary for response to various stresses. The GOLs (BnGOLS-1) activity was also positively correlated with desiccation tolerance in cabbage seeds (*Brassica napus*) during the vegetative growth period [8]. The tolerance was observed around 21-24 days after flowering cabbage, coinciding with the accumulation of raffinose and stachyose. The

BnGOLS-1 transcripts accumulation was concomitant with the formation of these two RFO members [8].

In *Arabidopsis thaliana* leaves overexpressing HsfA2 (Heat-Shock Transcription Factor A2; [49]) was also found highly induced GolS1, -2, -4 and Raffinose Syntase 2 (RS2; EC 2.4.1.82, highlighted in red in Figure 2) transcriptions. The galactinol and raffinose levels in the transgenic plants were higher compared to the wild-type lines, both in the control condition. These higher levels were positively correlated with an increase in plant tolerance when exposed to the studied stresses [mevalonate (50 mM), high salt (100 mM NaCl) and low temperatures (4°C)].

Latter, Pennycooke et al. [50] studied the expression of  $\alpha$ -galactosidase gene (EC 3.1.2.22 highlighted in red in Figure 2) from petunia (*Petunia x hybrida* "Mitchell"), monitoring acclimated plants to low temperatures (4°C) and in response to increasing temperature (25°C). Transcripts induction were observed after one hour of desacclimation occurring together with an increase in enzymatic activity and decreased raffinose content, suggesting that the rise in temperature can regulate the RFO catabolism of certain members, through gene regulation that encoding  $\alpha$ -galactosidase.

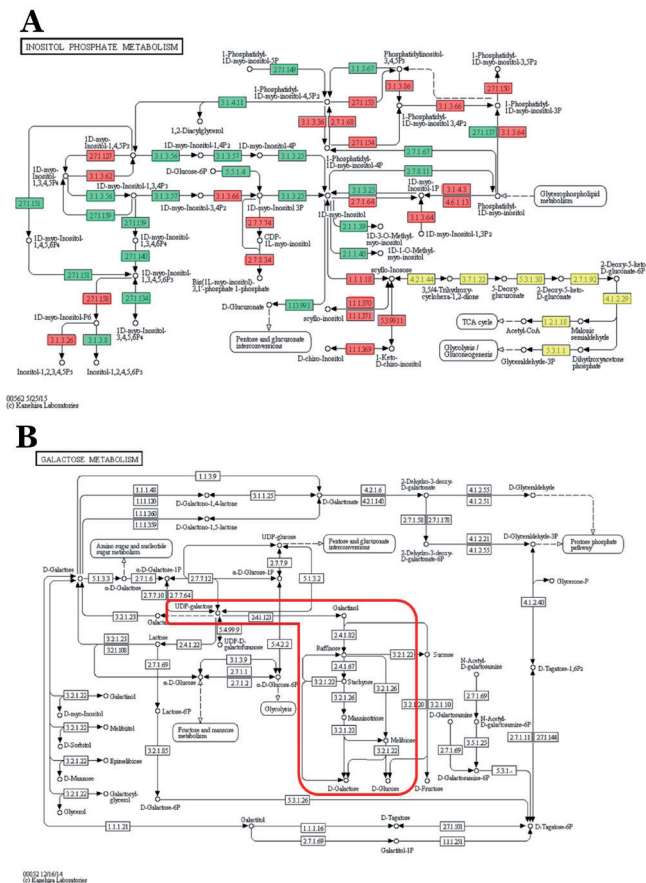
Thus, the diversity of functions performed by compounds presenting in the Ins metabolism was shown in the described works. Also, studies of distinct isoforms showed positive correlations with plant responses to various abiotic stresses. In this way, the identification of new transcripts, as well as the understanding of its regulation (spatial and temporal) in plants under unfavorable conditions for the development may lead to the discovery of new genes with biotechnological potential.

According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database (<http://www.genome.jp/kegg/pathway.html>), which provides diagrams of various metabolic processes, at least 45 enzymes are associated with the metabolic pathways described here. Of these enzymes, 21 (highlighted in green boxes in Figures 3A and 3B) have been studied in previous works addressing the transcriptional expression of their genes or effects on plants under stress. Therefore, there are at least 24 enzymes (in red boxes in Figures 3A and 3B) that have not been targets of these analyses, with significant potential for further research in biotechnology.

## 6. Ins and its derivatives in humans: Antinutrients *versus* disease prevention

Once Ins and its derivatives are present in vegetables and these are part of the daily diet of large populations around the world, it is essential to analyze their potential effects on consumers. The Ins and related metabolites play a heterogeneous physiological role, depending on the concerned organism, plant or animal (including human). In plants, as already mentioned, such compounds help regulate plant homeostasis during periods of stress. In animals (including humans), their influence has very diverse physiological repercussions. Initially, they were only seen as harmful agents because some representatives when present in certain

plants could act as antinutritional factors, thereby reducing the bioavailability of important nutrients and the nutritional value of the food. According to Kokhar and Apenten [51], this effect is present as a result of a selected adaptive mechanism due to a "chemical warfare" between higher plants and herbivorous pests.



**Figure 3.** KEGG Pathway database diagrams presenting: (A) Inositol Metabolism; (B) Galactose Metabolism, showing the Raffinose Family Oligosaccharides (highlighted in red). Green Box: enzymes with transcriptional modulation data available from stress assays. Red Box: enzymes without transcriptional modulation data available in the literature. Yellow Box: enzymes not covered in this review.

Among the various Ins-derivatives, phytic acid (1-D-myo-inositol-P6; Figure 1) is the most studied, concerning the impacts on human and animal health. Its unique structure provides the ability to chelate cations such as iron, zinc, potassium, magnesium, and copper, forming

insoluble salts denominated phytate. These salts adversely affect animal's ability to absorb and digest nutrients [52, 53]. Phytates can form complexes with proteins, changing their structures and their enzymatic activities and characteristics of solubility and proteolytic digestibility [54]. However, there are reports that show positive aspects in phytates consumption. The presence of these in the diet of patients with diabetes has positive effects in reducing the level of blood glucose due to decreased starch digestion rate and slowing of gastric evacuation [55]. There are also reports of activity against HIV replication, kidney stones prevention, reduction of cholesterol and triglycerides levels, as well as assistance in prevention of heart diseases (for review see [54]). Studies also indicate that both Ins [56] and phytic acid [56, 57] have anticancer properties. With regard to RFOs, besides the fact that they are potential antinutritional factors, there are indications that they may act as important immunostimulants in animals (including humans). Also, RFOs' involvement is suggested in universal mechanisms of oxidative balance in several taxa [58].

## 7. Concluding remarks and perspectives

Experimentally, mutants and transgenic analyses are being successfully carried out to uncover the various roles played by Ins-related compounds. It is known today that some phosphorylated derivatives of inositol are connected with a large number of signaling procedures which are regulated by both abiotic and biotic stress. Methylated and oxygenated Ins-derivatives, including RFOs, have also proven to be active agents in the process of plant acclimatization to unfavorable conditions, involved in a number of functions. However, there is a gap to be filled. About two dozen enzymes associated with the synthesis of these compounds have not been adequately studied and they represent an area of high biotechnological potential.

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# Effect of Salinity Stress on Gene Expression in Black Tiger Shrimp *Penaeus monodon*

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Additional information is available at the end of the chapter

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## Abstract

Penaeid shrimps are euryhaline in nature and have the ability to survive and adapt in a wide range of salinities (3–50 ppt). The shrimps are cultured under a variety of conditions in many tropical and subtropical countries. Osmotic and ionic regulation is an important mechanism of environmental adaptation in crustaceans. However, drastic changes in abiotic and biotic conditions result in stress to the shrimps during the culture period. Salinity and temperature are the two major environmental factors that have huge impact on culture shrimp, affecting their physiological and metabolic parameters, which in turn affect shrimp growth, molting, and survival. Changes in the abiotic factors, chemical and biotic factors result in reduced immunity of shrimp and vulnerability to bacterial and viral diseases. This chapter describes the effects of low and high salinity on the gene profile changes of black tiger shrimp *Penaeus monodon*, and the functional role of these genes in shrimp salinity stress is discussed.

**Keywords:** *Penaeus monodon*, Salinity stress, Differentially expressed genes

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## 1. Introduction

Penaeid shrimps being euryhaline can adapt to thrive and survive in a wide range of salinity conditions. Shrimps are, therefore, cultured and reared under different farming conditions in tropical and subtropical countries. Water quality management is an important criterion in shrimp farming for survival and growth of the shrimp. The optimal salinity conditions for penaeid shrimp ranges differently for different species. *Penaeus monodon*, which can tolerate low salinity of 5 ppt to high salinity conditions of 40 ppt has optimal range of salinity (15–25 ppt) for optimal growth [1]; juvenile *Penaeus chinensis* grows best at 20–30 ppt salinity range [2]; the optimal conditions of salinity for growth was estimated to be in the range of 22–34 ppt

for *Penaeus latissulcatus* [3]. *Penaeus semisulcatus*, an Indo-Pacific species requires higher salinity (30–35 ppt) for growth [4]. Best performance (growth, survival, total biomass) for *Penaeus indicus* PL20 and PL60 at salinities between 20 and 30 ppt was observed after acclimation period [5]. Highest increase in biomass and production was observed at 25 ppt for *Penaeus merguensis* [6]. *Litopenaeus vannamei*, a native species of the Pacific coast is a very important shrimp species, which is cultured under semi-intensive and intensive conditions in many parts of world. The juveniles of this species have optimal growth and survival in salinity range of 33–40 ppt [7]. It is also preferred for culture in low salinity water as it can tolerate very low salinities of 1–2 ppt.

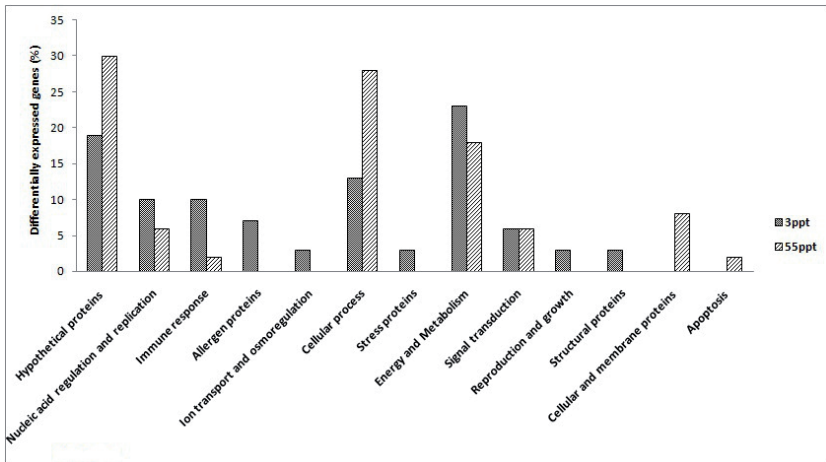
However, the variable climate conditions result in drastic changes in abiotic factors causing stress to the shrimps during the culture period, which influences culture of euryhaline penaeids. In summer months, there is increase in salinity in ponds due to high evaporation rates and in rainy season the salinity decreases. The marine crustaceans are generally osmotic and ionic conformers in nature. The shrimps hypo-osmoregulate above the iso-osmotic point and hyper-osmoregulate below the iso-osmotic point through a osmoregulation mechanism.

In crustaceans, gills which are highly permeable external surfaces are the primary sites involved in osmoregulation. In the larval stages of penaeids, the typical features of osmoregulatory epithelia are present in pleurae and branchiostegites [8]. The overall ion-transport and osmotic regulation process involves ions absorption or excretion between the external and internal medium through osmoregulatory organs, such as gills, the antennal glands that mainly function in urine production, and the gut in decapod crustaceans [9-10]

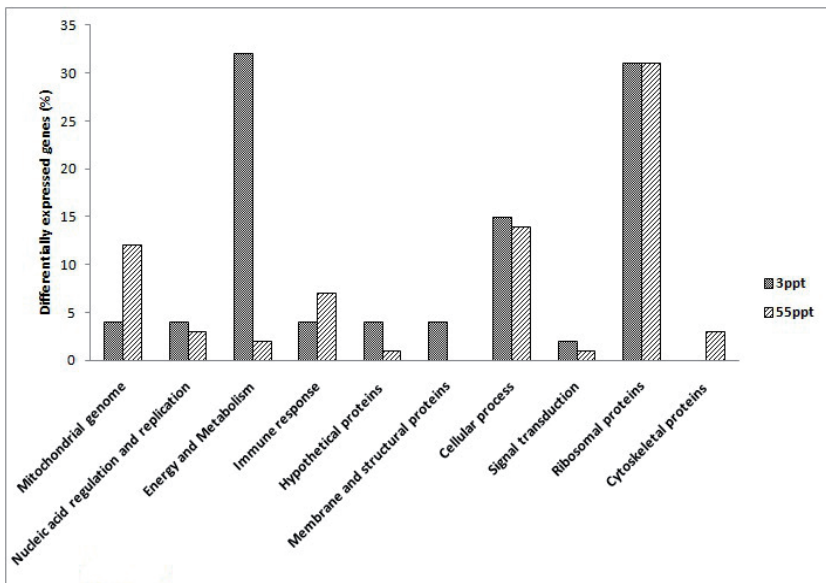
The crustaceans have two well-known important enzymes which are central to osmotic and ionic regulation and ion uptake. The transepithelial movement of monovalent ions requires the action of  $\text{Na}^+/\text{K}^+$ -ATPase or the sodium pump utilizing ATP as energy source. The other major enzyme involved in osmoregulation is transport-related enzyme carbonic anhydrase, which plays a role in producing  $\text{H}^+$  and  $\text{HCO}_3^-$  through catalysis of respiratory  $\text{CO}_2$ , for counterions in  $\text{Na}^+$  and  $\text{Cl}^-$  uptake. In penaeids, carbonic anhydrase is reported to be involved in both hyper- and hypo-osmotic regulation and is induced against low and high salinity exposure, indicating its role in ion uptake and excretion process [11].

In our study, we have constructed suppression subtractive hybridization (SSH) cDNA libraries to identify differentially expressed genes in shrimp *P. monodon*, in response to salinity stress. The SSH clones obtained from the forward SSH cDNA libraries (Figure 1) and reverse SSH cDNA libraries (Figure 2) constructed from gut tissues of shrimp exposed to low (3 ppt) and high (55 ppt) salinity on BLAST analysis, revealed several functional categories.

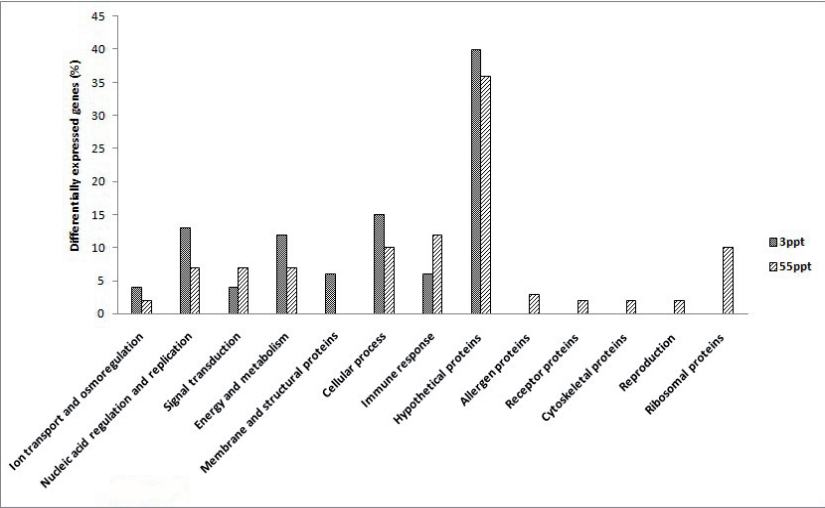
Similarly, we obtained several functional categories of genes from the forward SSH cDNA libraries (Figure 3) and reverse SSH cDNA libraries (Figure 4) constructed from gill tissues of shrimp exposed to low (3 ppt) and high (55 ppt) salinity conditions. These differentially expressed genes were subjected to RT-qPCR for gene expression analysis. Based on our study, we discuss herein in this chapter some of the important genes identified as differentially expressed in response to salinity stress in shrimp.



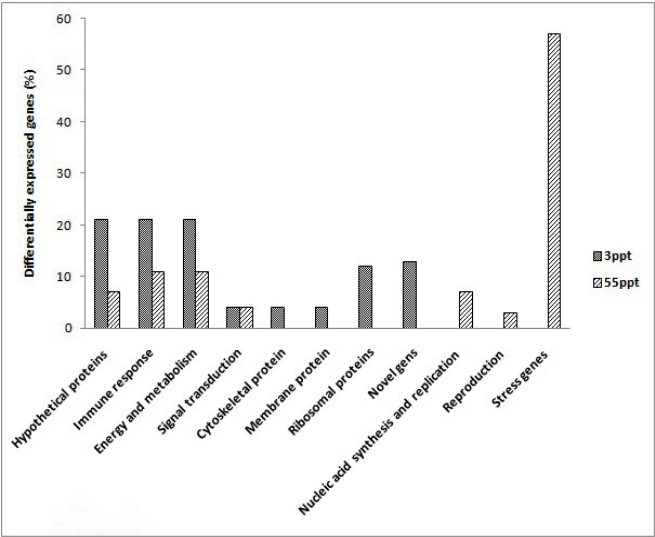
**Figure 1.** Differentially expressed genes from the forward SSH library of gut tissues of *P. monodon* under low (3 ppt) and high (55 ppt) salinity conditions



**Figure 2.** Differentially expressed genes from the reverse SSH library of gut tissues of *P. monodon* under low (3 ppt) and high (55 ppt) salinity conditions



**Figure 3.** Differentially expressed genes from forward SSH library of gill tissues of *P. monodon* under low (3 ppt) and high (55 ppt) salinity conditions



**Figure 4.** Differentially expressed genes from the reverse SSH library of gill tissues of *P. monodon* under low (3 ppt) and high (55 ppt) salinity conditions



## 2. Osmoregulatory genes

### 2.1. Na<sup>+</sup>/K<sup>+</sup>-ATPase

Na<sup>+</sup>/K<sup>+</sup>-ATPase, a transmembrane protein, contains three subunits,  $\alpha$ -,  $\beta$ -, and  $\gamma$ - subunit, which are involved in exporting three Na<sup>+</sup> from cytosol in exchange for two K<sup>+</sup> or NH<sub>4</sub><sup>+</sup> from extracellular fluid for each ATP hydrolyzed. The crustacean  $\alpha$ -subunit, which is 71–74% identical in amino acid sequence to those of vertebrate  $\alpha$ -subunit sequences, binds to ATP and functions for the catalytic action of the enzyme [12]. The binding of Na<sup>+</sup>/K<sup>+</sup>-ATPase complex to basolateral membrane requires participation of  $\beta$ -subunit and the  $\gamma$ -subunits of enzyme [13]. The activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase in gill tissues of crustaceans depends on the osmoconcentration gradient occurring between hemolymph and the external medium. In crustaceans, there is an increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity when transferred from natural seawater to dilute seawater [14–15]. With the lowering of salinity when compared to that of normal seawater, the euryhaline crustacea undergo hyperosmoregulation. Increased enzymatic activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase and increased  $\alpha$ -subunit gene expression has been observed in the gill tissues of crabs [12]. Substantial increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase specific activity (300%), Na<sup>+</sup>/K<sup>+</sup>-ATPase protein levels (200%), and gene expression level of  $\alpha$ -subunit (150%) has been observed in blue crab *Callinectes sapidus* crabs during acclimatization to dilute seawater of 10 ppt salinity [16]. *P. monodon* reared in 7 ppt seawater showed drastic morphological alterations of the antennal glands. The shrimps also showed higher expression and activity of the enzyme Na<sup>+</sup>/K<sup>+</sup>-ATPase in the antennal glands under low salinity conditions [17]. *L. vannamei* when transferred to different low salinity conditions ranging from 15 ppt to 1 ppt revealed no significant difference within 3 h for Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit gene expression and enzyme activity. However, there was a rapid increase at 6 h followed by decrease in the expression level from 12 h to 24 h suggesting Na<sup>+</sup>/K<sup>+</sup>-ATPase is stimulated by salinity stress [18]. The study involving V-H ATPase  $\alpha$ -subunit and Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\beta$ -subunit response to environmental stress (bacteria, pH, Cd, salinity, and low temperature) revealed both the genes to be responsive to these environmental stress conditions. However, the V-H ATPase  $\alpha$ -subunit and the Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\beta$ -subunit, which is involved in proper folding and transport of Na<sup>+</sup>/K<sup>+</sup>-ATPase enzyme, were found to be more sensitive to salinity stress when compared to other stress factors. The exposure of *L. vannamei* to salinity stress resulted in significant changes in the expression of V-H ATPase  $\alpha$ -subunit and Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\beta$ -subunit gene expression levels in the hepatopancreas and gills of the shrimp. Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\beta$ -subunit gene expression after exposure to 5 ppt increased to a highest level (17-fold) at 12 h in the gill tissues, whereas, in hepatopancreas the maximum gene expression levels (4.4-fold) were observed 6 h after exposure to 10 ppt salinity conditions [19].

At low (3 ppt) salinity conditions stress conditions, significant increase in the Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit gene expression levels was observed in gill (34.28-fold) tissues of *P. monodon* [20]. At higher salinity stress of 55 ppt, *P. monodon* Na<sup>+</sup>/K<sup>+</sup>-ATPase gene responded to salinity stress conditions with significant expression levels in gill (15.23-fold) tissues [21]. These results suggests that *P. monodon* Na<sup>+</sup>/K<sup>+</sup>-ATPase gene is involved in osmoregulatory process in shrimp and responds significantly under salinity stress.

### 3. Immune genes

Crustins, belonging to family of antimicrobial peptides (AMPs) are shown to be differentially expressed in response to various immunostimulants and microbes [22]. Different isoforms of crustins have been isolated from a variety of penaeid shrimps [23-25]. The up-regulation of crustin-like AMP in shrimps suggests functional role of AMPs primarily in the shrimp immune response [26]. Under hyperosmotic stress, crustinPm5, crustinPm1, and crustin-like Pm gene levels were found to be up-regulated [27]. In our study, we observed crustin gene was up-regulated in all the three shrimp tissues of gills, gut, and muscle analyzed at low salinity stress [20]. Hence, crustins having antimicrobial activity also functionally respond to salinity stress in shrimps.

Another class of AMPs, the penaeidin gene was found to be down-regulated in gill, gut, and up-regulated in antennal gland tissues of shrimp exposed to high salinity stress (55 ppt) conditions [21]. These changes in the gene expression levels of penaeidin may be regulated through variations in hemocyte numbers in salinity-stressed shrimp. Antibacterial proteins such as lysozymes are involved in nonspecific innate immunity in shrimps [28]. Significant up-regulation of lysozyme gene expression occurred in gill (16.25-fold) tissues of shrimp exposed to low salinity stress [20]. In these shrimps, the anti-lipopolysaccharide factor, which is considered to play an important role in shrimp immune response [29], was significantly down-regulated in gill and gut tissues as compared to up-regulation in the muscle tissues of shrimp.

In general, as a result of environmental stress, the expression of immune-related shrimp genes transcripts gets affected [30]. The shrimp exhibits reduction in immune parameters when exposed to salinity stress conditions, leading to decreased resistance against pathogens [31-32]. White shrimp *L. vannamei*, when transferred to low salinity conditions showed significant decrease in hemocyte count, phenoloxidase activity, respiratory burst, and superoxide dismutase activity, which further reduced on challenge with *Vibrio alginolyticus*. Hence, innate immunity in shrimps reduces with combined effect of low salinity and bacterial challenge [32].

## 4. Cellular-process-related genes

### 4.1. Ubiquitin-conjugating enzyme

The ubiquitin proteolytic system is involved in various cellular processes such as cell cycle regulation, cellular response to stress, and immune response [33-34]. In shrimps, the ubiquitin-conjugating enzyme E2r (UBE2r) gene isolated from *Marsupenaeus japonicus* showed that the gene expression level changed significantly in the developing testis and ovary with higher level in the testis than in the ovary, which indicates the functional role of UBE2r in oogenesis and spermatogenesis of shrimp [35]. The gene expression pattern of ubiquitin in abdominal muscle of 3-month-old shrimps *L. vannamei* at different molt stages on examination did not increase significantly in premolt stages and was found to be relatively stable at all stages of

the molt cycle [36]. Antiviral function has been demonstrated with ubiquitin-conjugating enzyme, E2, isolated from *F. chinensis*, which could inhibit WSSV replication and ubiquitinate WSSV RING domain-containing proteins [37]. In *P. monodon* ubiquitin-conjugating enzyme E2, gene was up-regulated in muscle and gill tissues under low salinity stress conditions [20]. In American lobster, *Homarus americanus* the polyubiquitin gene expression revealed significant changes in abdominal muscle and hepatopancreas during osmotic stress, indicating that proteins may be more susceptible to ionic fluctuations [38].

## 4.2. Cathepsins

The proteases are distributed into four major classes: aspartyl proteases, metalloproteases, serine proteases, and cysteine proteases [39]. The cathepsins belonging to cysteine proteases in addition to cellular protein degradation and turnover are also involved in numerous other physiological processes. In fishes, the cathepsin La isoform has been implicated in yolk-processing mechanism during oogenesis and embryogenesis [40]. In shrimps (*Metapenaeus ensis*), cathepsin L, with predominant expression in hepatopancreas, is suggested to have a role in food digestion. The immunolocalization of cathepsin L in the nucleus of oocyte suggests its specific physiological role in shrimp oocytes [41]. The cathepsin L-like proteinases isolated from *L. vannamei* showed that specific activity and mRNA expression of cysteine protease were associated during the molt cycle [42]. The expression of the cathepsin C gene expression in hemocytes of *L. vannamei* could be induced after *V. alginolyticus* challenge, which reached a maximum level at 4 h post challenge period, indicating that the cathepsins in shrimp may be involved in immune response [43]. The aspartic protease cathepsin D obtained from *M. japonicus* and *P. monodon* were characterized for physical and chemical properties and were shown to have identical subunits and similar optimal pH [44]. The analysis of cathepsin B gene expression in *L. vannamei* tissues revealed high expression levels in midgut gland and gut, which are involved in food digestion process. The changes in the gene expression levels and enzymatic activity were induced by starvation in the midgut gland of the white shrimp [45]. Significant increase in the cathepsin B gene expression levels in gills, gut, and muscle tissues of shrimp exposed to low (3 ppt) salinity conditions suggest that *P. monodon* cathepsin gene responds to the shrimp adaptive mechanism to salinity stress [20].

## 5. Signal transduction genes

### 5.1. 14-3-3 protein

The members of the 14-3-3 protein family are dimeric proteins that are expressed in a wide range of organisms and tissues. They are involved in modulation of protein interactions through phosphorylation process. The other diverse functional roles include interaction with a large number of protein kinases, DNA, Raf kinase, and regulation of cell cycle progression [46]. In plants, 14-3-3 protein activates and regulates plasma membrane H<sup>+</sup>-ATPase through fusicoccin responsive system [47]. Two Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit forms were detected in the gill transcripts of crab *Pachygrapsus marmoratus*, which differed in presence of an 81-nucleotide

sequence near the translation start site in the D form when compared to the C form. The extended D form was found to contain the binding motif for the regulatory protein 14-3-3, suggesting that Na<sup>+</sup>/K<sup>+</sup>-ATPase may be stimulated by this regulatory protein binding [48]. The two isoforms of 14-3-3 proteins identified from the *P. monodon* were shown to have varied gene expression profiles during salinity adaptation in response to hypo-osmotic (3 ppt) or hyper-osmotic (40 ppt) salinity stress conditions. Induction of 14-3-3B gene expression in gills of shrimp acclimated to low salinity water suggests that it is likely to be involved in controlling osmoregulation in *P. monodon* under hyperosmotic conditions [49]. The up-regulation of the 14-3-3 gene expression in the tissues of shrimp exposed to low (3 ppt) or high (55 ppt) salinity conditions suggests that *P. monodon* 14-3-3 gene may have a potential role in shrimp response to salinity stress [20-21].

## 5.2. Calreticulin

Calreticulin, a versatile lectin-like chaperone and important endoplasmic reticulum luminal resident protein, is involved in Ca<sup>2+</sup> homeostasis and molecular chaperoning. Calreticulins, which are highly conserved in most of the eukaryotes, are involved in the synthesis of various molecules and in many other biological and physiological processes of an organism. The highest expression of calreticulin was detected in ovary of *F. chinensis*. The gene expression varied at different molting stages and could be induced by heat shock and WSSV challenge, indicating multifunctional role of calreticulin [50]. The *P. monodon* calreticulin showed changes in expression profile in response to high-temperature stress, indicating its potential as a biomarker for stress responses in shrimps [51]. Calreticulin also responds to salinity stress with significant increase in the gene expression levels in gills and muscle tissues of *P. monodon* [20].

## 5.3. Innexins

Innexins, which are members of large multigene families, are involved in formation of gap junctions for cell-to-cell communication [52]. In crustaceans, innexin expression has been associated with developing lobster stomatogastric nervous system [53]. In *P. monodon*, the transcripts of innexin-2-like protein showed increased expression in response to yellow head viral disease [54]. Innexin-2 was found to be more abundantly expressed in testes than ovaries of *P. monodon*, indicating functional role of innexin-2 in spermatogenesis but not in oogenesis [55]. The high gene expression observed for innexin 2 with 14.43-fold in muscle tissue of shrimp under low salinity stress of 3 ppt indicates gap junctions regulation during salinity stress in shrimps [20].

# 6. Energy and metabolism genes

## 6.1. Arginine kinase

Arginine kinase plays a major role in energy metabolism and is a phosphotransferase that catalyzes the reversible transfer of phosphate from phosphoguanidine to ADP, resulting in

generation of ATP [56]. In shrimps, Penm2 allergen gene having conserved guanidino specificity region and showing very high sequence similarity with crustacean arginine kinase has been isolated and characterized from *P. monodon* [57]. In crustaceans, such as in blue crab *Callinectes sapidus*, the arginine kinase gene expression is associated with salinity changes. The arginine kinase flux reduced under hyperosmotic treatments and increased with the hypo-osmotic treatments [58]. *M. japonicus* when subjected to severe hypoxic stress revealed up-regulation of arginine kinase indicating metabolic response of arginine kinase under hypoxic stress [59]. Arginine kinase was found to be differentially expressed and up-regulated in WSSV-infected blue shrimp (*Penaeus stylirostris*) [60] and the gene expression could be induced against LPS immunostimulation in *L. vannamei* indicating its correlation with immune response in shrimps [61]. The differential expression of arginine kinase in gills, gut, and muscle tissues of shrimp exposed to low (3 ppt) salinity conditions indicates that arginine kinase plays an important role in metabolic process associated with salinity stress in crustaceans [20].

## 6.2. Ferritin

Ferritin plays a functional role in iron storage and metabolism. In shrimps, this large multi-functional and multisubunit protein gene has been isolated and characterized from *L. vannamei*, which revealed that ferritin is expressed in most of the tissues of shrimp with major expression in hemocytes [62]. The administration of ferritin resulted in increased immune response in *L. vannamei* with enhanced survival rate in WSSV-challenged shrimps and maintained physiological homeostasis of shrimps [63]. In *M. rosenbergii*, the isolated ferritin gene showed conserved domain for the ferroxidase center and the administration of iron enhanced expression of ferritin gene in a tissue-specific manner [64]. The recombinant ferritin was shown to confer protection in *P. monodon* infected with *Vibrio harveyi* [65]. The expression of ferritin mRNA could be induced with heavy metal ions  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  and WSSV challenge in *F. chinensis* [66]. The gill tissues of *P. monodon* when subjected to low (3 ppt) salinity stress revealed significant increase in ferritin gene expression (8.79-fold), indicating its functional role in salinity stress in shrimps [20].

## 6.3. Intracellular fatty-acid-binding proteins

Intracellular fatty-acid-binding proteins (FABPs) are lipid-binding proteins that help in transport of fatty acids across extra- and intracellular membranes and are involved in various other biological processes such as modulation of signal transduction; gene transcription, especially of lipid metabolism; and cell growth and differentiation [67]. FABPs have been well-characterized in vertebrates as compared to that in invertebrates. In crustaceans, the FABP cDNA having fatty-acid-binding motifs has been cloned and characterized from the freshwater crayfish *Pacifastacus leniusculus* and *P. monodon* [68]. The activity of specific  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Cl}^-$  ion channels are regulated by various fatty acids. These ion channel regulations may be carried out directly through fatty acid interactions [69]. The shrimp (*P. monodon*) gut tissue revealed highest gene expression level of FABP (14.05-fold) at high salinity stress conditions (55 ppt) and at low salinity stress conditions (3 ppt) with 13.30-fold; the osmoregulatory process may therefore involve the FABPs in shrimps [20-21].

#### 6.4. Acyl-CoA binding protein

Acyl-CoA binding protein (ACBP) is a highly conserved protein. In yeast it is involved in transportation of acyl-CoA esters from the fatty acid synthetase to acyl-CoA-consuming process [70]. The protein, which was first identified in mammals, acts as a neuropeptide that prevents binding of diazepam/endozepine to GABA receptor system [71-72] and is also involved in regulation of several acyl-CoA-dependent processes [73]. In addition, ACBP is involved in many other functions, which include regulating biosynthesis of fatty acid, functional regulation of enzymes and genes, intracellular acyl-CoA pool regulation, acyl-CoA esters donation required for  $\beta$ -oxidation and vesicular trafficking [74], and in regulation of m-calpain [75]. In plants *Arabidopsis thaliana*, different types of ACBPs are encoded [76], such as the ACBP1 and ACBP2, which are membrane-associated proteins [77-80]; ACBP3, which is the extracellularly targeted protein [81]; and ACBP4, ACBP5, and ACBP6, which are the cytosolic proteins [82-83]. ACBPs are involved in abiotic stress tolerance in plants. The ACBP2 in *Arabidopsis* was shown to be involved in heavy metal (Cd) tolerance [84-85]. ACBP6 and ACBP1 are functionally involved in increased freezing tolerance [78],[86]. In shrimp, ACBP functions in antibacterial [87] and antiviral response [88]. The ACBP gene in *P. monodon* was identified to be differentially expressed in the SSH libraries constructed from the gut tissues of both low (3ppt) and high (55ppt) salinity stressed shrimps. The complete cDNA sequence of ACBP consisted of 273 bp ORF coding for 90 amino and showed ligand-binding conserved domains similar to the other members of ACBP family. At 2 weeks post 3 ppt salinity stress conditions, a significant increase in the ACBP transcripts expression was observed in gills and muscle tissues with highest levels in the gut tissues (28.08-fold). Similar increase in the gene expression levels was observed in shrimps exposed to high salinity stress conditions of 55 ppt in gills and muscle tissues with gut tissues revealing high (11.95-fold) levels of gene expression [89]. These results suggest a functional role of ACBP gene during salinity stress in shrimps.

#### 6.5. Catechol-O-methyltransferase

O-methyltransferase (OMT) is an enzyme found in a wide range of organisms such as microbes [90], where it is involved in antibiotic biosynthesis [91], and in fungi, where it is involved in biosynthesis of aflatoxins [92]. The OMT found in plants are well characterized for their functional role in O-methylation during biosynthesis of lignin, stress resistance, and disease tolerance [93]. In crustaceans, farnesoic acid O-methyltransferase (FAMeT) catalyzes farnesoic acid methylation resulting in production of isoprenoid methyl farnesoate, which is involved in metabolic and physiological regulation [94]. Catechol-O-methyltransferase (COMT), which is a type of O-methyltransferases, helps in catalyzing the transfer of methyl group to the hydroxyl group of catechol compounds from S-adenosyl-L-methionine. In higher animals, the COMT helps in catalysis of methylation of various macromolecules that are involved in different functional and regulatory purposes and is present in soluble and membrane-bound forms [95]. In shrimps, the construction of suppression subtractive hybridization (SSH) libraries from *P. monodon* gill tissues resulted in identification of COMT gene as one of the differentially expressed genes subjected to salinity stress. The COMT gene was differentially regulated in both the SSH libraries generated from low and high salinity conditions. The ORF of COMT gene of 666 bp size revealed the coding protein with 221 amino acids [96]. The *P. monodon* COMT showed the conserved domains present in superfamily of S-adenosylmethio-

nine-dependent methyltransferases, which includes COMT, CCoAOMT family of indolethylamine N-methyltransferase from humans, and OMT from *Bacillus subtilis* [97]. The *P. monodon* COMT was found to be up-regulated in low and high salinity stress conditions at different time intervals in shrimp tissues (gills, guts, and muscles), suggesting a functional role of this gene in salinity stress tolerance in shrimps [96].

## 7. Stress genes

### 7.1. Heat shock proteins

Heat shock proteins (HSPs) initially discovered in *Drosophila melanogaster* are a highly conserved set of polypeptides present in both prokaryotic and eukaryotic cells. They are generally involved in conferring thermotolerance as molecular chaperones by refolding the denatured proteins and also respond against various other stresses. They play a crucial role in organisms' response to heat shock and cellular stress. In addition, the HSPs are also important for cellular damage protection and in maintaining cellular homeostasis [98]. In aquatic animals, the HSPs respond to environmental pollutants, abiotic stress, and are involved in disease resistance against viral and bacterial pathogens. The functional role and significance of HSP in farmed aquatic animals is demonstrated in stimulating the immune response [99]. In shrimp (*P. monodon*), some of the HSPs such as HSP21 have been characterized for gene expression against WSSV infection [100]; HSP70 gene expression was found to increase in the shrimp hemocytes after heat shock treatment [101]; and HSP90 gene expression has been related with the ovarian maturation [102]. The transcriptional levels of HSP21, HSP70, and HSP90 were inducible under the heat shock and responded upon bacterial exposure in *P. monodon* [103]. Expression of HSP70, which is one of the widely studied HSP in aquatic organisms, was high during short-term hyperthermic treatment when compared to hypoxic and osmotic stress in *P. monodon* [104]. The significant increase in gene expression level HSP21 in the gut and muscle tissues of *P. monodon* exposed to low salinity stress (3 ppt) conditions, indicates its functional role in osmotic stress in shrimps [20].

In conclusion, the construction of SSH cDNA library in response to low (3 ppt) and high (55 ppt) salinity stress in shrimp (*P. monodon*) led to identification of various differentially expressed genes. The significant up-regulation expression of several genes at transcription level in gills, gut, antennal gland, and muscle tissues of shrimp in response to two-week post-salinity stress condition indicates their functional role in gene pathways and regulatory mechanism of osmotic stress at the molecular level.

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# Functional Genomics of Biotic and Abiotic Stresses in *Phaseolus vulgaris*

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Additional information is available at the end of the chapter

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## Abstract

Common bean is the most important legume for human consumption in the world, being a crop extremely diverse in cultivation methods, uses, range of environments in which it is adapted, morphological variety, among others. Besides its high demand and production, this crop is threatened by a series of biotic and abiotic adversities during its life cycle, which leads to losses in yield of up to 100%. In this chapter, we explored the main constraints that affect common bean and the ways this plant reaches tolerance or resistance to them, highlighting studies at the molecular level that enabled to understand the mechanisms by which common bean perceives, responds, and adapts to a stress condition. Special focus has been given to the most recent findings in the understanding of the mechanisms underlying drought tolerance and anthracnose resistance. Thereby, we reviewed some genetic and functional genomic studies concerning the genes and pathways involved in each case. Furthermore, we outline important genetic resources of *Phaseolus vulgaris*, as well as the technologies and methods used toward these findings.

**Keywords:** Common bean, anthracnose, drought, genetic resources, gene expression

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## 1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important legume crop for consumption worldwide [1]. It is cultivated in a range of crop systems and environments, being Latin America the leading producer and consumer, where beans are a traditional and significant food source, especially in Brazil, Mexico, the Andean Zone, Central America, and the Caribbean [2]. As a source of protein, folic acid, dietary fiber, and complex carbohydrates, common

beans are considered nutritionally rich and when consumed as part of the diet can lead to an increase in the use of maize and rice proteins since their amino acids are complementary [2]. They are also a good non-meat source of iron, providing 23–30% of the daily recommended levels of this element in a regular adult diet [2–3].

In Latin America, Africa, and Asia, common bean is primarily a small farmer crop cultivated with few purchased inputs and is subject to a large amount of biological, edaphic, and climatic issues [2–4]. Conditions under which common beans are regularly cultivated in this regions are extremely variable [3], and such factors coupled with the highly specific local preferences for seed characteristics (size, shape, color) have been challenging to establishing the breeding strategies in accordance with what is needed.

Beans from these regions usually present low yielding [2], since they are frequently cultivated employing low to non-mechanized irrigation systems. Common bean is mostly grown in drought-prone areas, and long-term drought exposure periods seems to be a global and endemic threat affecting the majority of the production areas [4]. It has been observed that common bean is particularly susceptible to drought especially during the flowering and grain-filling stages (R5 and R8, respectively) [5, 6]. Moderate levels of water deficit usually lead to a reduction in plant biomass, lower seed number per pods, earlier maturation, lower seed yield and weight, and reduction in nitrogen fixation [7].

Not only abiotic factors but also several biotic constraints represent a significant threat to common bean cultivation. Fungi, bacteria, viruses, and nematodes cause a series of diseases, concurring for the death of some plants or even significant areas from whole plantations, causing a severe reduction in yield. Examples of such diseases are rust, white mold, anthracnose, root rots, bacterial blights (halo, yellow, common), powdery mildew, mosaic viruses, etc. Environmental conditions (temperature, soil moisture) and management practices (varieties, crop rotation, irrigation, and chemical control) may prevent the establishment of some diseases and reduce losses, but for some of them the most appropriate strategy for controlling consists on the development of resistant varieties and high-quality seeds.

This chapter is especially driven to describe the most recent developments in the understanding of the molecular mechanisms involved in drought tolerance and anthracnose resistance. In that purpose, we outline important genetic resources of *Phaseolus vulgaris*, as well as the technologies and methods used toward these findings.

## 2. Genetic resources

### 2.1. Center of origin and domestication of common bean

Beans belong to the Fabaceae family (Leguminosae, Papilionoidae) and genus *Phaseolus*. About 55 species of *Phaseolus* are described but only five are cultivated: *P. vulgaris*, *P. acutifolius*, *P. lunatus*, *P. polyanthus*, and *P. coccineus* [8].

*P. vulgaris* is naturally distributed in a wide area from northern Mexico to northeastern Argentina. High morphological diversity has been found among wild populations of *P.*

*vulgaris* from one to the other extreme of the geographical distribution of the species [9, 10]. This variability is observed in different leaf shapes, growth habits, flower colors but especially for seeds in terms of colors, shapes, and sizes [10]. This variability has also been observed at the molecular level, with several molecular marker studies such as with microsatellites [11–15], AFLP [14–16], and SNPs [17–20].

Several of these studies recognized two major ecogeographical gene pools of wild beans: Mesoamerican and Andean. However, the geographic structure of the wilds reveals more complexity, with an additional third pool between Peru and Ecuador, characterized by a particular storage seed protein, phaseoline type I [21, 22]. Further examinations showed wild populations from Colombia to be intermediates. A marked geographic structure in populations from the Mesoamerican pool has also been described [23, 24]. Originally, the population from northern Peru and Ecuador was considered an ancestral population from which *P. vulgaris* originated. From this core location, beans probably were spread north and south, resulting in the Mesoamerican and Andean pools, respectively [22, 25, 26].

Nevertheless, based on several studies [27–29], there has been a discussion over an alternative and older hypothesis which considers that ancestral beans were distributed through Mesoamerica. The high genetic diversity encountered within these gene pools has been used to support this hypothesis. Furthermore, the Mesoamerican origin of the common bean has been suggested based on sequence analysis of data from five small gene fragments [32]. A whole-genome comparison among 30 individuals from each Mesoamerican and Andean wild populations showed high genetic differentiation among gene pools and, a demographic inference for the Andean gene pools, suggested it was derived from a Mesoamerican population with only a few thousands of individuals [20]. Nevertheless, the debate on the origin of the species remains and more studies are on their way to better understand the core center of origin of common bean.

Likewise, the domestication process of *P. vulgaris* has been another matter of debate and extensive molecular studies. Initially, morphological and enzyme profiles showed the existence of two major centers of bean domestication: Mesoamerica and Andean, encompassing six races [10]. There are indications that nearly 8,000 years ago common bean was independently domesticated in Mexico and South America [30–33]. Domestication was followed by local adaptations resulting in landraces with different characteristics [20]. However, much more has yet to be deciphered and the recent application of genomic approaches is promising to a better understanding of the domestication processes of common bean and other crops [34].

## 2.2. Core collections

The high diversity of common bean has been collected in germplasm banks in which those are not only kept but also constantly improved, generating new genetic materials by adding new combinations obtained through many crosses and new generated populations. Several bean germplasm collections are available, but some of the core collections that must be highlighted here are held at the Centro Internacional de Agricultura Tropical (CIAT), in Cali, Colombia. Information on every wild and domesticated beans from this collection may be obtained in the website <http://isa.ciat.cgiar.org/urg/main.do?language=en>. Another core collection is from the

United States Department of Agriculture (USDA), found on <http://iapreview.ars.usda.gov>. Brazil has held a very significant collection of landraces and domesticated beans at EMBRAPA Arroz e Feijão and also at the Agronomic Institute of Campinas, which has been developing several new commercial varieties (<http://www.iac.sp.gov.br/areasdepesquisa/graos/feijao.php>). Much more details about bean collections are found on Genesys (<https://www.genesys-pgr.org/welcome>), a portal to information about Plant Genetic Resources for Food and Agriculture, describing many bean accessions and the places where they are kept. These collections comprise a very rich source of genetic materials that possess several features to be exploited in functional genomic and molecular breeding studies for the species. Among the genetic resources available are wild beans, landraces, breeding lines, recombinant inbred populations, all distinguished between the Andean and Mesoamerican gene pools.

### 2.3. *Phaseolus vulgaris* – The genome

A recent publication showed the work that has been done for many years to sequence the genome of the common bean, whose assembly has been made public by a consortium between the USDA-NIFA project “A sequence map of the common bean genome for bean improvement” and DOE-JGI and ARRA (*Phaseolus vulgaris* v1.0 – <http://phytozome.jgi.doe.gov/>). In total, 472.5 Mb of the 587-Mb genome were assembled and 98% of the sequence were genetically anchored on the 11 chromosomes, using a SNP high-density map (7,015 markers) genotyped in the RIL (recombinant inbred lines) population derived from the cross Stampede × Red Hawk and another map with 261 SSRs and a set of Infinium markers. The 472.5 Mb were arranged in 41,391 contigs (~9.32% gap) and the annotation revealed 27,197 total protein-coding genes and 31,638 protein-coding transcripts, resulting in 4,441 total alternatively spliced transcripts [23]. The publication of this genome opened a series of new resources for developing research in many fields such as the mechanisms involved in biotic and abiotic stresses in common bean.

## 3. Identification of genes involved with anthracnose resistance

The pathogenic system *Colletotrichum lindemuthianum*/*Phaseolus vulgaris* has been studied as a model for almost one century [36] and, its infection mechanisms and disease development were extensively studied in the 1980s [37, 38, 39]. This species of *Colletotrichum* is one of the most studied due to its economic importance, infection strategy [38], ease of *in vitro* cultivation [40], and availability of an efficient and reproducible transformation system [41]. As a model system for plant/fungi interaction, it can provide valuable information in several aspects, like plant defense responses, phytoalexins, fungal-degrading cell wall enzymes, differentiation of fungal infection structures.

The susceptible common bean cultivars establish an interaction of compatibility with this fungus, what allows the development of the anthracnose disease, strongly affecting production and yield of beans; furthermore, this fungus has great variability and many races identified [42, 43]. With this, the genetic resistance is an important way of disease control. Genetic studies

indicate that the common bean resistance to the anthracnose is related to multi-allelic loci [44, 45], which mostly comprise dominantly inherited genes denominated *Co* [45]. Bean cultivars resistant to anthracnose containing *Co* gene (s) respond to pathogen inoculation with an incompatible interaction. This interaction initiates with the pathogenic fungus inoculation, causing physiological variations and rapid changes in gene expression that activate defense responses in the host plant. Necrotrophic points, typical of a hypersensitive reaction (HR), occur at the infection site, resulting in a limited fungal growth. The HR, considered the primary response of the plant to the pathogen attack, is characterized by an oxidative burst due to the formation of reactive oxygen species (ROS) [46]. This initial plant response can be considered definitive in the determination of resistance to the pathogenic agent.

In the compatible interaction, the establishment of the pathogen in the plant tissue is aided by the production, by the fungus, of virulence effectors induced by the host [47, 48]. The life strategy adopted by the fungus (hemibiotrophic) make infected tissues remain without outward symptoms for up to three or more days [49, 50], and only after the entrance in the necrotrophic phase cause plant cell death and emergency of pathogenic lesions.

Despite the multi-allelic resistance already described for the common bean, new sources of resistance should always be searched due to the high variability among pathogen populations and occurrence of newly evolved virulent races. Furthermore, knowing the molecular pathways involved with the process of resistance in the plant can enable the transference of important genes to susceptible cultivars.

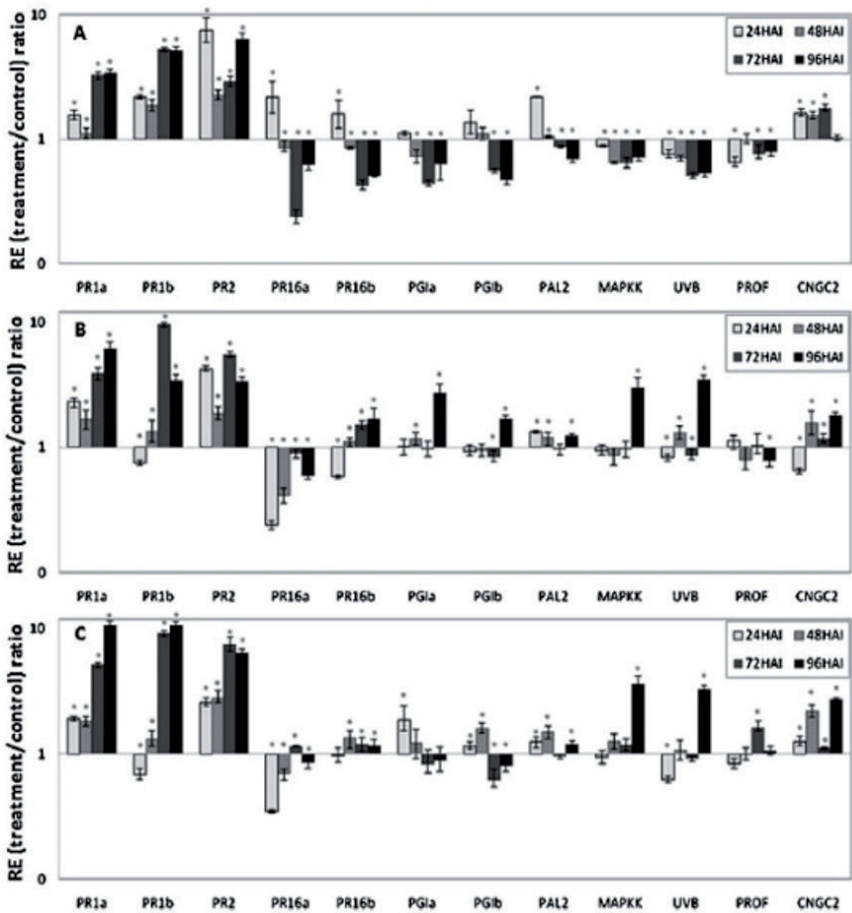
Common bean is not a species prone to be genetically transformed, although there is already a transgenic cultivar resistant to the Golden Mosaic Virus [51]. Furthermore, the genome of common bean was made available only recently, and reverse genetics through the use of mutant lines is still difficult due to few resources. Then, transcriptomic analysis appears as a suitable method to investigate the changes in gene expression in a plant under any kind of stress.

### 3.1. Gene expression profiles from an incompatible interaction

Studying gene expression profiles of incompatible interactions between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum* may be an advantageous strategy to identify genes involved with anthracnose resistance because it can provide a direct answer about the potential modulations occurring in metabolic processes during an infection event with a resistance response by the host.

The first study devoted to generate a unigene data set of common bean using ESTs sequencing was described by [52], through the analysis of three EST libraries from the cultivar SEL 1308, consisting of 19-day-old trifoliolate leaves, 10-day-old stem shoots, and 13-day-old stem shoots inoculated with the race 73 of *C. lindemuthianum* in an incompatible interaction. At that time, a total of 5,255 ESTs were sequenced, 2,332 from inoculated stem shoots, with 1,583 unigenes assigned for this library. More recently, [53] used this database to select candidate genes based on the number of ESTs found per unigene (or tentative contig) in each library, to study expression profiles in temporal and spatial scales during fungus infection. Twelve genes were

chosen and tested in leaves, hypocotyls and epicotyls inoculated with *C. lindemuthianum* (Figure 1).



**Figure 1.** Relative expression (RE) ratio of 12 pathogenesis resistance-related transcripts in leaves (A), epicotyls (B), and hypocotyls (C) of common bean genotype SEL 1308 at 24, 48, 72, and 96 HAI with the race 73 of *C. lindemuthianum*. Non-inoculated tissue was used as control for expression levels to determine the RE ratio. The symbol \* above the bars indicates statistical significance calculated using the Pair Wise Fixed Reallocation Randomization Test with  $P \leq 0.05$ . (Extracted from [53]).

All genes showed modulation during this incompatible interaction. Some of them were rapidly activated and kept this activation, like PR1a, PR1b (known as good molecular markers for SAR (systemic acquired resistance)), and PR2 (a b-1,3-glucanase) (Figure 1), which act in plant

defense by hydrolysing the cell walls of the fungal pathogens. All the others showed a variety of expression patterns according to time and tissue, for instance, PR16 proteins (germin-like), which were upregulated early in leaves and then fall down, and in epicotyls and hypocotyls only PR16b was upregulated in late periods of analysis (Figure 1). This kind of study not only give us an idea of the kinetics of induced defense responses of common bean against the anthracnose fungus but also can be used as a base line for others studies of resistance against a broad range of pathogens [53]. Furthermore, this work revealed differential and specific transcriptional profiles in different tissues of common bean, where specific defense processes may occur to contain the development of a pathogen. For more details, see [53].

### 3.2. The immune system model for *Phaseolus vulgaris*/ *Colletotrichum lindemuthianum*

The innate immunity is a primitive way of defense against microbial infection shared by plants, insects, and animals. Differently from mammals that have mobile cells specialized in defense, each plant cell is responsible for its own defense. Thus, each cell integrates environmental signals in order to activate local and systemic defense responses.

The same EST libraries described before [52] were used by [54] to investigate global changes in gene expression of *P. vulgaris* inoculated with *C. lindemuthianum* in an incompatible interaction. In an extensive bioinformatics analysis, the ESTs were aligned by tBLASTX with the *Arabidopsis thaliana* (L.) Heynh genome, which is completely annotated and curated. With this, it was possible to conduct a functional comparison between the fungus-inoculated and the mock-inoculated library. Figure 2 shows the overall mechanisms found in this study. It was found that some processes involved with plant-pathogen interaction were upregulated in common bean in response to the presence of fungus, like defense response to fungus (GO: 0050832), regulation of defense response GO:0031347), regulation of response to stress (GO: 0080134), and stomatal movement (GO:0010118).

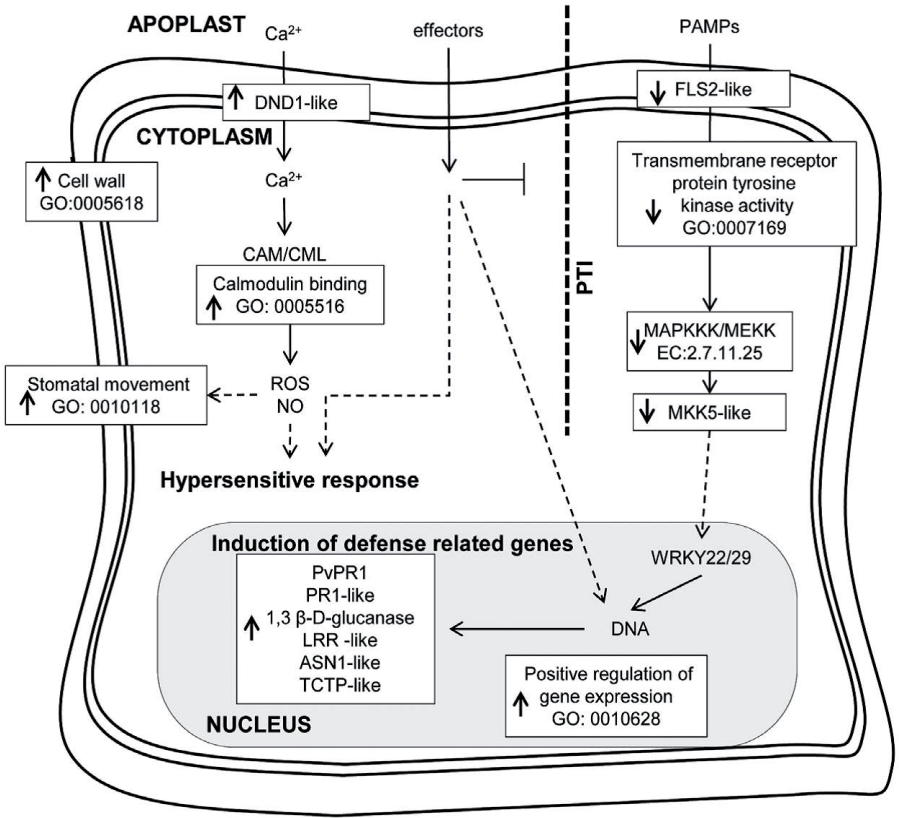
Response to cytokinin stimulus (GO:0009735) and ethylene-mediated signaling pathway (GO: 0009873) were upregulated, while jasmonic acid biosynthetic (GO:0031408) and metabolic (GO: 0009694) processes, as well as response to gibberellin stimulus (GO:0009739) and abscisic acid-mediated signaling pathway (GO:0009738) were downregulated, indicating that there may be a hormonal control and cross-talk in common bean defense against *C. lindemuthianum*. According to [54], hormonal mechanisms can be used in some pathosystems for resistance and in others for susceptibility depending on the fungus life-style. While jasmonates (JA) were found to be important in disease susceptibility in *Arabidopsis* and tomato infected with *Pseudomonas syringae* [55, 56], a biotrophic bacterium, in common bean it is not used in signaling since *C. lindemuthianum* is a hemibiotrophic pathogen.

Still based on the analysis of ESTs libraries, infected common beans have its metabolism modulated for detoxification from ROS burst, once HR is occurring during the incompatible interaction; also, a downregulation of genes was observed related to plant development (organelle fission (GO:0048285), cell cycle process (GO:0022402), pattern specification process (GO:0007389), post-embryonic morphogenesis (GO:0009886), and regulation of post-embryonic development (GO:0048580), typical of plants under stress that needs to reallocate resources to defense responses.



Finally, transcripts encoding for cell wall proteins showed an increase in abundance, suggesting that activities as cell wall modification, pathogen recognition, and transport and secretion of defense compounds are important in bean defense against anthracnose.

When looking for molecular components of the plant innate immunity (PTI – PAMP-triggered immunity or ETI – effector-triggered immunity), [54] observed that ETI (characterized by HR) can negatively regulate PTI. Transmembrane receptor protein tyrosine kinases and MAPKKK/MEKK transcripts were significantly downregulated in fungus-inoculated library and this data validate by RT-qPCR.



**Figure 2.** A model of the bean innate immune system. The proposed model represents key molecular components and metabolic processes known to be involved in plant-pathogen interactions. Gene Ontology (GO) categories and Enzyme Codes (EC) inside boxes are differentially represented in the bean EST libraries (arrowheads pointing down represent downregulation and arrowheads pointing up represent upregulation). Continuous arrows represent established relationship between components of the pathway and intermittent arrows represent undirected relationship. Components of PAMP-triggered immunity (PTI) are depicted to the right of the diagram. (Extracted from [54]).



## 4. Identification of genes involved with drought tolerance

### 4.1. Gene expression profiles from Subtractive Libraries of cDNA and RT-qPCR

Long-term global climate changes have conducted to an increase in the occurrence of drought episodes in different locations around the globe [57, 58]. This fact concurrently with agriculture expansion into marginal areas have led to increasing environmental instability, a limiting factor for crop yielding with potential negative impact on food stocks worldwide. This problem is especially aggravated by the rapid human population growth and consequent augmented food demand, especially in developing countries. Therefore, drought has been considered one of the main abiotic constraints that affect agriculture [59].

Plant responsiveness to drought stress can be affected by different factors; it mainly depends on the severity of the event, including the extension of the water-deficit period, and if the plant has already been exposed to a previous regime of acclimatization to this condition [60]. Acclimatization to drought results from a series of integrated events that comprehend the perception of the stress by the plant, translation of the signal, the regulation of the expression of specific genes, and the consequent shifts at metabolic level [61].

Drought perception often leads to a reduction in the photosynthetic rates of the plant, affecting its growth, which is directly related to shifts in carbon and nitrogen metabolism [62]. This reduction on the photosynthetic net is a result of a series of coordinated events such as stomatal closure and the reduction on photosynthetic enzymes activity [63, 64]. At cellular level, drought stress results in the accumulation of the chemically reactive molecules containing oxygen termed as ROS (reactive oxygen species), which ultimately can also drive to the oxidative stress of the photosynthetic apparatus [65,66], thus ROS-efficient removal for avoid oxidative stress can be used as a measure for drought stress tolerance in plants [67]. These molecules act inside cells as secondary messengers involved in signaling transduction that leads to specific stress responses [65]. At molecular level, some specific sets of genes can undergo different processes of regulation of their expression (mainly through cycles of induction and repression of expression) determining new protein synthesis profiles, therefore changing their biological functions [61]. Several genes have been both collectively and individually implicated in drought stress response in plants, but the identification of which ones would be more useful for adoption at breeding and transformation approaches aiming the improvement of drought stress tolerance remains a great challenge [68, 69].

Strategies for plant transformation and genetic breeding usually focus on the transfer of a single or a small set of genes that can codify for specific biochemical pathways or for final targets of the signal transduction pathways that usually are controlled by constitutively active promoters [70]. These gene products protect the plant against the damages caused by drought stress and are divided into different classes: osmoprotectors (amino acids, dimethyl-sulfonyl compounds, mannitol, sorbitol, complex carbohydrates); enzymatic and non-enzymatic ROS scavengers; LEA proteins; heat-shock proteins; ion transporters; fatty acid desaturases; aquaporins; signaling components (homologous to histidine kinases, MAP kinases, Ca<sup>2+</sup>-dependent protein kinases, protein phosphatases, Ca<sup>2+</sup> sensors, inositol kinases); transcription

factors (EREBP/AP2, bZIP, ABRE, NAC, MYB); and growth regulators (ABA, cytokines, brassinosteroids) [60–71, 72].

At the transcriptional level, expressed sequence tags (EST) sequencing has been widely used to discover and identify genes potentially involved in drought stress response [73, 74]. Therefore, by using a great amount of transcriptome profiling methods, researchers are being able to contrast genotypes with different potential for drought tolerance, thus increasing the already large datasets of candidate genes for using in studies regarding the improvement of drought stress in plants.

Suppressive subtractive hybridization (SSH) method has been successfully used to construct cDNA libraries enriched in transcripts that are differentially expressed in target tissues, developmental stages, and specific treatments in various biological systems [74,75]. The SSH method [76] consists on the hybridization of one cDNA population (*tester* – sample whose genetic profile is of interest, e.g., drought-tolerant genotype), with an excess of cDNA from a control population (*driver* – usually drought-susceptible genotype or well-watered control), followed by the separation of the nonhybridized molecules (*target genes* – the ones of interest) from the hybridized ones (what is common for both samples). In this session, we are aiming to present some of the results obtained by our group during the construction of a SSH library contrasting populations of cDNAs extracted from root tissues of two common bean genotypes, BAT 477 (*tester* – drought-tolerant) and Carioca 80SH (*driver* – drought-susceptible), both submitted to a 192 hours of water-deficit regime at the R5 developmental stage [77].

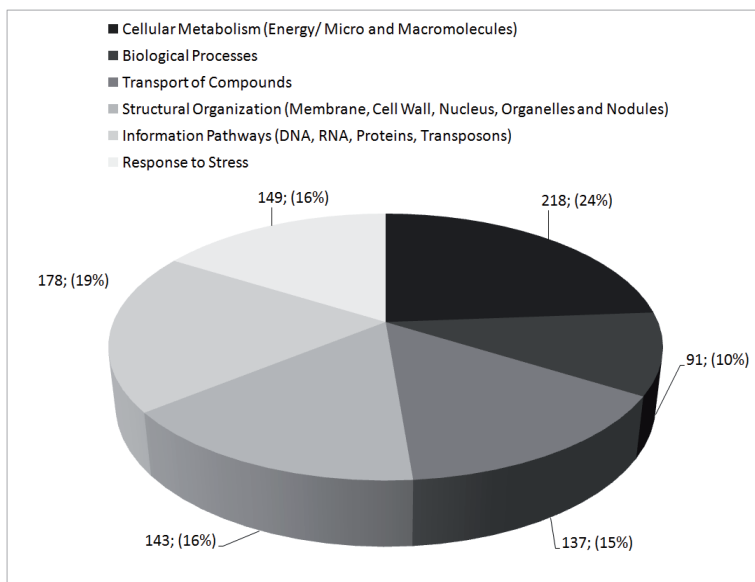
The sequencing of the SSH library consisting of a BAT 477 cDNA population enriched for transcripts exclusively expressed by this drought-tolerant genotype under 192 hours of water-deficit generated 1,572 valid reads that were grouped into 189 contigs and 931 singletons (total of 1,120 unigenes). Public green plant EST databases (available at the National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/>) and bioinformatics tools were used for initial trimming, clustering formation, gene annotation. Final functional annotation was achieved using the Gene Ontology Consortium database (<http://geneontology.org/>) combined to the CS model (*CombinedScheme*) developed by [78] (<http://www.biochem.ucl.ac.uk/~rison/FuncSchemes/>) (for further details on adopted bioinformatics tools and analysis specifications, see [77]).

Gene annotation based on homology search using the BLASTX tool and redundant sequences with E-value  $\leq e^{-5}$  generated putative information on 896 reads: 315 reads displayed similarity with sequences with not yet assigned putative or hypothetical functions, and 259 reads had good quality control but had no similarity with sequences available in public databases. Table 1 lists the most abundant contigs annotated via BLASTX tool and classified under the biological process that they might be involved in the plant. Final functional annotation classification of the 896 reads is summarized in Figure 3. The six main functional classes are described as follows: 1. Cellular Metabolism (Energy, Macro/ Micronutrients); 2. Biological Process (Cell Division, Regulation, Signaling, Cell Death, Signal Transduction, and Nuclear Cycling); 3. Transport of Compounds; 4. Structural Organization (Membrane, Cell Wall, Nucleus, Organelles, and Nodules); 5. Information Pathways (DNA, RNA, proteins, and transposons); and 6. Stress Response (Biotic and Abiotic Stresses).

Access code in library	Number of reads	GI number	Description/ Species	e-value
<i>Cellular Metabolism (Energy/Micro and Macromolecules)</i>				
Contig147	3	255579310	pyruvate decarboxylase, putative [Ricinus communis]	4e-80
Contig7	3	83283965	malate dehydrogenase-like protein [Solanum tuberosum]	e-171
Contig23	3	255638912	glyceraldehyde-3-phosphate dehydrogenase [Glycine Max]	e-119
Contig28	3	255540625	glutaredoxin-1, grx1, putative [Ricinus communis]	2e-40
<i>Biological Processes</i>				
Contig123	3	224094081	spliceosomal complex, [Populus trichocarpa]	3e-35
Contig171	3	75304713	Methionine adenosyltransferase, [Phaseolus lunatus]	1e-83
Contig79	4	156181612	S-adenosylmethionine decarboxylase [Phaseolus vulgaris]	3e-25
Contig127	4	75304713	Methionine adenosyltransferase, [Phaseolus lunatus]	5e-90
<i>Abiotic Stress Response</i>				
Contig74	4	42571665	interferon-related developmental regulator family protein [Arabidopsis thaliana]	6e-53
Contig105	3	192910730	light-inducible protein ATLS1, [Elaeis guineensis]	2e-30
Contig14	3	75708857	group 3 late embryogenesis abundant protein, [Phaseolus vulgaris]	6e-23
Contig61	3	806310	proline-rich protein, [Glycine max]	7e-18
Contig37	4	1732556	LEA5 [Glycine max]	3e-34
Contig97	4	1350522	LEA protein [Picea glauca]	3e-27
Contig24	9	1732556	LEA5 [Glycine max]	3e-34
<i>Biotic Stress Response</i>				
Contig3	3	184202203	isoflavone synthase 1 [Vigna unguiculata]	1e-85
Contig3	3	184202203	isoflavone synthase 1 [Vigna unguiculata]	1e-85
Contig17	9	130835	PvPR2 [Phaseolus vulgaris]	1e-79
<i>Transport</i>				

Access code in library	Number of reads	GI number	Description/ Species	e-value
Contig164	3	61651606	plastidic phosphate translocator-like protein1 [Mesembryanthemum crystallinum]	1e-61
Contig80	4	255587991	cation:cation antiporter [Ricinus communis]	1e-39
Contig2	3	255552798	ATP binding protein, putative [Ricinus communis]	8e-30
Contig64	4	255637247	calcium ion binding [Glycine max]	2e-38
<i>Structural Organization (Membrane, Cell Wall, Nucleus, Nodulation and Organelle)</i>				
Contig142	3	255549412	Vesicle-associated membrane protein, putative [Ricinus communis]	8e-31
Contig137	3	146233385	abscisic acid ABA receptor [Populus trichocarpa]	1e-24
Contig148	3	194466205	putative L24 ribosomal protein [Arachis hypogaea]	2e-23
Contig11	5	255584772	histone h2a, putative [Ricinus communis]	2e-27
Contig19	3	57013900	NitaMp027 [Nicotiana tabacum]	6e-33
Contig83	4	30682545	ARF3 (ADP-Ribosylation factor 3) [Arabidopsis thaliana]	1e-59
<i>Information Pathways (Processing of DNA, RNA and proteins/ Transposons)</i>				
Contig154	3	187940303	NAC domain protein [Glycine max]	8e-84
Contig51	4	20138704	eIF-5A [Manihot esculenta]	7e-40
Contig52	4	255646048	transferase activity [Glycine max]	2e-58
Contig162	3	155212489	N3 protein [Glycine max]	1e-47
<i>Unclassified</i>				
Contig72	3	255626205	unknown [Glycine max]	3e-78
Contig87	3	255639776	unknown [Glycine max]	3e-71
Contig98	3	255647862	unknown [Glycine max]	8e-55
Contig145	3	255646578	unknown [Glycine max]	5e-47
Contig6	4	224101339	predicted protein [Populus trichocarpa]	5e-30
Contig64	4	255637247	unknown [Glycine max]	2e-38
Contig77	4	255637264	unknown [Glycine max]	2e-10
Contig82	6	255629893	unknown [Glycine max]	7e-27

**Table 1.** List of most abundant contigs containing the original ID of SSH library, number of reads assigned, NCBI identification number (GI) of the EST used for gene putative annotation inference, EST description and correspondent species, e-Values. ESTs are organized according to the functional class Biological Process [77].

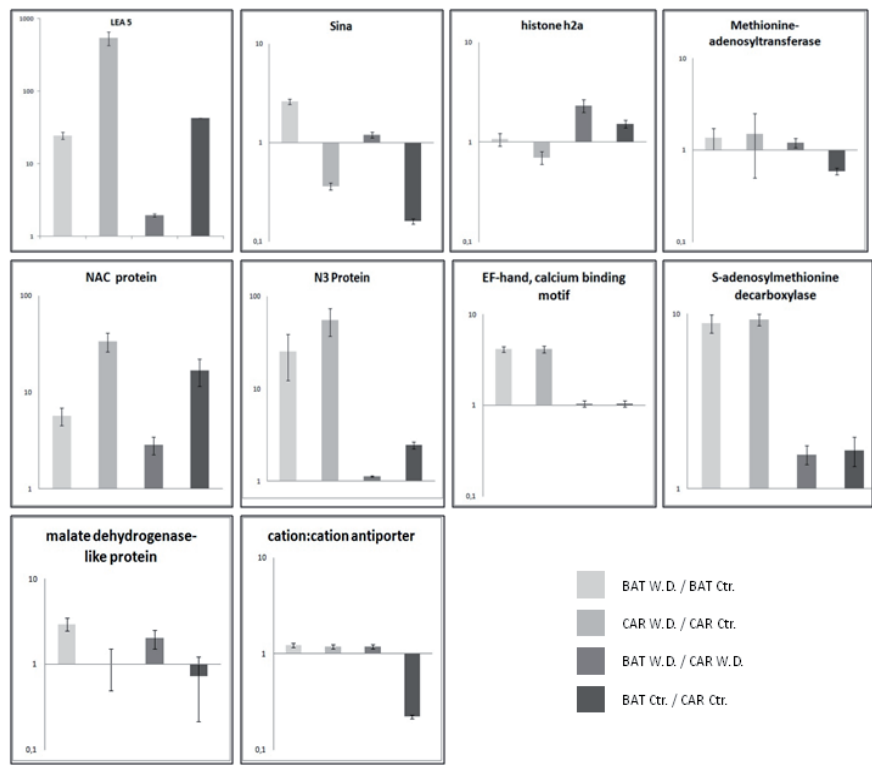


**Figure 3.** Final functional annotation classification of the 896 reads with positive putative gene description information (E-value  $\leq e^{-5}$ ) obtained after blastx homology search using NCBI EST green plant public database (<http://www.ncbi.nlm.nih.gov/>). Each sector contains the relative EST numbers, in parenthesis the representation percentage in relation to the total number of ESTs successfully annotated. (Extracted from [77]).

The most abundant functional class was Cellular Metabolism (218 ESTs), something that was already expected since, as mentioned before, plants that undergo long periods of water deprivation tend to reduce its photosynthetic rates due to shifts in carbon and nitrogen metabolism, therefore needing to adjust its basal metabolic rates in order to keep homeostasis. Such elevated number of ESTs may be related to a more efficient mechanism of metabolic adjustment present in the drought-tolerant genotype BAT 477 that allows these plants to better adapt during the drought period, thus achieving better survival rates. And, 148 reads were grouped at the Response to Stress and some of them may be directly linked to drought stress tolerance: transcription factors (NAC, DREB, ABRE, WKRY, bZIP, MYB), transmembrane transporters like aquaporins,  $K^+/H^+$  pumps and  $Ca^{+2}$  transporters, osmoregulators (LEA proteins, dehydrins, proline-rich peptide chains), and proteins associated with protection (heat-shock proteins, chaperones) and degradation (ubiquitins) [77].

A common bias usually associated with the SSH library construction technique combined with the traditional Sanger-based sequencing technique [79] is the possibility of obtaining false-positives. Recently, the use of SSH library technique combined with new high-throughput NGS-sequencing technologies [74–80, 81] has provided evidence for solving this issue since they are more able to achieve sample saturation. In RNA-Seq technologies, saturation could be reached when an increment in the number of reads does not result in additional true

expressed transcripts being detected or in more features called as differentially expressed when two or more conditions are compared [82]. However, the elevated costs usually associated with NGS-sequencing technologies make further experiment validation a more attractive option for researchers. The validation experiments consist of taking the same RNA samples initially used for cDNA library construction and re-analyzing them using a complementary technique, usually microarrays (for those species who already have this platforms available) [83,84] or RT-qPCR (quantitative reverse transcription PCR) [85].



**Figure 4.** Relative gene expression profile of 10 ESTs selected from the BAT 477 drought stress-related SSH library [77]. Captions: BAT – BAT 477 drought-tolerant common bean genotype; CAR – Carioca 80 SH drought-susceptible common bean genotype; W.D. – 192h of water-deficit treatment; ctr. – control plants. BATWD/BATctr (BAT 477 under stress compared with BAT 477 control plants); CARWD/CARctr (Carioca 80SH under stress compared with Carioca 80SH control plants); BATWD/CARWD (BAT 477 under stress compared with Carioca 80SH under stress – SSH library initial condition); and BATctr/CARctr (BAT 477 control plants compared with Carioca 80SH control plants). (Extracted from [77]).

For the BAT 477 drought stress SSH library, it was selected as a set of 10 ESTs among those with most abundant contigs: *LEA5*, *Sina*, *histone h2a*, *methionine adenosyltransferase*, *NAC protein*,

*N3 protein, EF-hand – calcium binding motif, S-adenosylmethionine decarboxylase, malate dehydrogenase-like protein, cation:cation antiporter*. For each of the ESTs, a specific pair of primers for RT-qPCR analysis was designed [77] and gene relative expression quantification was obtained for the same tester and driver samples used for the SSH library construction (Figure 4). These results served well for the SSH library validation since all the selected transcripts revealed to be upregulated in BAT 477 plants under drought stress. Besides, for some of the transcripts (*LEA5*, *NAC protein*, *N3 protein*, *Ef-hand – calcium binding motif*, and *S-adenosylmethionine decarboxylase*), although they are expressed in lower concentrations on Carioca 80SH 192h drought-stressed plants, when compared to Carioca 80 SH controls, they undergo an even greater upregulation in relation to BAT 477 (Figure 4). This not only confirms the relevance of these transcripts on drought stress response regulation in common beans but also reveals that the drought-tolerant genotype BAT 477 may already keep a basal level expression of some important drought-related transcripts, thus stress perception by this drought-tolerant genotype may trigger more efficient signaling mechanisms that leads to a more discreet gene expression upregulation allowing the plant not to dislocate resources that otherwise may be saved for keeping homeostasis and therefore secure development and growth during the stress period.

#### 4.2. DREB transcription factors as candidates for drought-tolerance improvement

Finding candidate genes and investigating their functional role and association with drought-tolerance traits and mechanisms have been of prime interest for many crop plants such as common bean. The DREB transcription factors subfamily has been studied in depth as candidate genes for breeding of abiotic stress tolerance. This group comprises a series of genes intermediating the regulation process to cope with abiotic stresses effects such as drought. They were originally described by [86], which identified a *cis*-acting regulatory element, DRE (dehydration responsive element), present in the gene promoter COR78/RD29A and involved in the response to drought, high salinity, and low temperature, further named as DREB (DRE-Binding). These proteins are capable of binding to DRE to activate the expression of genes of the stress signaling pathway. DREB transcription factors are unique to plant species and so far several genes have been described in *Arabidopsis* and other plants [87, 88].

The primary feature of a DREB transcription factor is the presence of a highly conserved protein domain, the EREBP/AP2. It was discovered within *APETALA2*, which plays an important role in flowering and seed development in *Arabidopsis*. Several proteins have been found containing this domain along their amino acid chain, consisting of a repeated motif of approximately 60 amino acids [89–91]. All these proteins are comprised in the larger superfamily EREBP/AP2 divided into three families referred as AP2, ERF, and RAV, based on their sequence similarity and the number of EREBP/AP2 domains [92]. The ERF protein family contains only one EREBP/AP2 domain and is subdivided into two main subfamilies, CBF/DREB and ERF [91]. The amino acids 14 and 19 of the EREBP/AP2 domain distinguish DREBs (valine and glutamic acid, respectively) from ERF (alanine and aspartic acid, respectively) [91]. In addition, ERF genes are involved primarily in responses to biotic stresses such as pathogenesis while DREB genes have main role in abiotic stresses responses.

DREB genes can be divided into six subgroups (A-1 to A-6). This categorization was based on phylogenetic trees as well as particular features related to their induction. The two most studied groups have been A-1 and A-2. Genes *DREB1/CBF* belong to subgroup A-1 and have been characterized as induced by low temperature in *Arabidopsis* [93], but other studies revealed some inducibility under drought and salinity as well [91, 94]. *DREB2* genes are primarily involved in responses to osmotic stress (dehydration and salinity) [91, 95].

Most of DREB findings have been associated with *Arabidopsis*; however, many studies have been performed with other species as well, revealing several new orthologs and different inducibilities for each one of the six DREB subgroups. Some of these findings have been done with legumes such as *Medicago truncatula* and *Glycine max*, close relatives to common bean.

Few studies have been published so far for common bean DREB genes, and they were mostly related to polymorphic sites identification along gene sequences. Ref. [96] categorized two orthologs *DREB2A* and *DREB2B* and identified polymorphisms between some Mesoamerican and Andean genotypes. Further investigation of these genes has been done to identify polymorphism patterns across wild and domesticated common beans. An attempt for phenotypic associations with drought-tolerance traits has been performed as well, but no clear patterns were obtained [18].

The research team of University of São Paulo, Brazil, has been studying DREB genes in depth. A pre-categorization study of the *PvDREB* gene subfamily has been done [97], showing putative DREB representatives for the species. Several genes have been isolated and their expression profiles determined under several abiotic stresses, including drought. One particular gene showed strong induction under many abiotic treatments, such as drought, salinity, and cold [98]. Some genes have been selected for a deeper molecular basis understanding as well as for their functional role in improving drought tolerance as well as other abiotic stresses.

Some other studies have found DREB genes in whole transcriptome profiles, such as in one experiment contrasting the drought-tolerant cultivar Long 22-0579 and the sensitive Naihua, in which a RNA-seq analysis was performed for samples under drought and control conditions. DREB transcription factors were identified to be differentially expressed and RT-qPCR analyses showed one transcript had the relative number of transcripts increased during the drought period [99]. Moreover, not only drought treatments have been analyzed but also one transcriptome profile has been done for a salt-tolerant bean cultivar named Ispir. It revealed several AP2/EREBP genes differentially expressed when contrasting a saline hydroponic solution with control conditions. Nevertheless, authors have not performed further categorization to identify which of those genes fitted *PvDREB*-specific characteristics [100].

Much more has to be done with DREB genes in common bean. Isolating and characterizing DREB genes for the species seems to be an important step toward the improvement of beans for abiotic stresses tolerance, especially for drought.



### 4.3. Phenotyping for drought tolerance in common bean

The identification of genomic regions or candidate genes, their functional role, and association with drought tolerance in common bean are fundamental aspects to understand the molecular signatures involved in acquiring such tolerance. However, in that purpose phenotyping methods are essential to effectively proving the effect of those genes on traits of interest. Thereby, it is important establishing and standardizing a phenotyping methodology to compare and select genotypes with different levels of stress tolerance in the studies one might be conducting. Furthermore, bringing data from the lab and greenhouse to the field is a big challenge, but of great importance for successfully applying the knowledge obtained about the genes, genotypes, and phenotypes of interest.

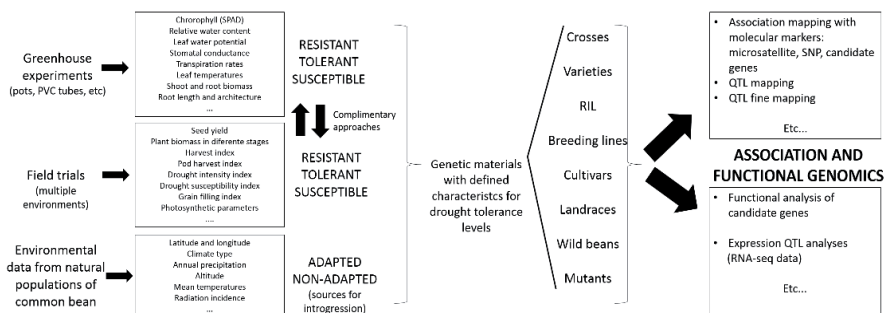
Phenotyping techniques have been developed to differentiating common bean accessions and cultivars for their levels of drought tolerance. Greenhouse trials have been applied to phenotype several shoot and roots traits and a common method employed has been the soil tube screening system assay that has been developed at CIAT [101]. Ref. [102] points out several traits that might be measured through such system, including many photosynthetic traits (photosynthetic efficiency, total chlorophyll content – SPAD, stomatal conductance, transpiration rates, leaf temperatures, leaf water potential), shoot and root biomass at the time of harvesting, leaf area and root traits (length, diameter, specific root length, and dry weight). Determination of root length might be done by image analysis system (WinRHIZO, Regent Instruments Inc.) [102] or might be manually determined by following root development on a graded plastic transparent tube in which plants were grown, all placed in PVC tubes.

The tube system developed by [101] was used to evaluate the effect of drought stress on root growth and distribution and compare different genotypes. Due to the difficulties of phenotyping roots in the field, this method has been shown to be a good complementary strategy applied in greenhouse conditions [102]. Examples in this sense are the studies of [103, 104] that analyzed the rooting patterns in greenhouse conditions with PVC soil cylinders and photosynthetic and yielding traits in different field areas. A population of recombinant inbred lines (RIL) from the crossing between the deep-rooting genotype BAT 477 and the small red-seeded and drought-susceptible DOR 364 was evaluated in both conditions. The greenhouse experiment showed that BAT 477 had significant larger root system based on root volume and deeper rooting ability, larger and thicker root, wide root diameter and biomass, under well-watered and progressive drought stress treatments [103].

For experiments conducted at the field, several traits can be evaluated since initial plant growth still harvesting. Ref. [102] made a very elaborated list with many parameters such as plant biomass at mid-pod filling and at harvesting time, seed yield, harvest index (HI), pod harvest index (PHI), drought intensity index (DII), and drought susceptibility index (DSI). The latter is based on the mean yields of a given genotype in drought stress and under no stress [102]. It assumes that one genotype will be more drought tolerant if the yielding is not so much reduced by the stress treatment in comparison to other genotypes. Pod harvest index has also been shown as a good indicator of drought tolerance, as shown by a field study in Ethiopia with the population from the crossing SXB 405 (breeding line) × ICA-Bunsi (white pea bean). Sensitive

lines presented significant reduction on PHI while no differences were observed for the most resistant lines [105].

Despite the availability of traits that might be evaluated in field conditions, the environment turns out to be a critical component interfering with results from one site to another. Drought field trials performed with the RIL population of the crossing BAT 477 × DOR 364, previously referred to the greenhouse experiment, showed significant variability across four locations evaluated [104]. A QTL analysis associating the field traits to a previous set of molecular markers disposed in a linkage map [106] showed significant QTL–environment interactions. Therefore, determining if one cultivar is tolerant to drought does not necessarily mean it will respond well to all environments, in a sense that it must be tested in multiple environments to check for its performance.



**Figure 5.** Strategies for phenotyping common beans for drought tolerance. Greenhouse experiments and field trials are complimentary strategies for evaluating several parameters and defining drought-tolerance levels. Recurring to wild beans based on environmental data might also be useful to select for beans adapted to drought episodes. Those evaluations altogether may be used toward the development of new varieties and the identification of genomic regions associated with the phenotypes evaluated as well as the functional role of candidate genes that are under characterization.

Although greenhouse and field methods have been developed to identify drought-tolerant genotypes and gene markers associated to such parameters, recent efforts have also been focused on the identification of sources of drought tolerance in wild beans spanning the natural area of distribution of *P. vulgaris* [107]. However, reliable estimations of drought tolerance in wild beans are not easy to establish, and attempts toward the development of new methods have been in course. Potential evapotranspiration models coupled with precipitation regimes were used to define a drought index for a series of wild bean accessions. Considering this factor along with the population structure might be a useful tool to analyze the levels of drought tolerance and use these materials for introgression of alleles of interest [107].

All these methods might be useful to carefully understand the phenotypic basis of drought tolerance variation in common bean genotypes. With standardized methods for the traits one might be interested, the accuracy between the association of molecular data and phenotypes might be much higher. It may be applied to QTL and association mapping studies, which link genome-wide molecular markers such as microsatellites, SNP, and gene-specific markers to

drought-related traits [103, 104, 106, 108]. On the other hand, standard greenhouse parameters can be used to test transgenic lines for determined candidate genes to verify their performances under imposed drought stress. Figure 5 shows a scheme of how greenhouse, field, and wild environment phenotyping studies might be useful for association and functional genomic studies in common bean.

## 5. Perspectives on the functional genomics of common bean

As mentioned before, common bean is not a species amenable for genetic transformation with the aim to test genes and to do functional studies. Thus, genomic mapping, transcriptomic and proteomic studies in contrasting genotypes, phases of development, different treatment/growth conditions, etc. are currently the most used approaches to identify genes linked to determined loci, verify changes in plant metabolism, and ultimately identify candidate genes suitable for molecular breeding or functional analyses.

The “omics” technologies and bioinformatics tools for large-scale data analysis have become essential to understanding the molecular systems that underlie various plant functions [109]. Despite common bean has been receiving increasing edible and economic importance, an investigation at a comprehensive omics level has been lacking in comparison to other model legume crops. As the genome sequences of *P. vulgaris* has become recently available, a new chapter has been opened for research with this crop. The genome release has provided a great miscellany of candidate genes that should be useful to improve common bean toward several different goals and approaches.

When considering abiotic stresses, some interesting NGS-related transcriptome data associated to drought [99] and salt-stress tolerance [100] as well as proteomic data related to drought [110], chilling [111], and osmotic stresses [112] have already been accessed. The consequential integration of a wide spectrum of omics data sets is then essential to promote translational research to engineer plant systems in response to the emerging demands of humanity.

Nevertheless, there is a big lack of information regarding interaction among stress sources. A recent trend for other crops has been the study of the effects of combined stress treatments such as drought versus salt, drought versus heating, drought  $\times$  salt  $\times$  nutrition, among others. These new studies try to represent most appropriately what really happens in the field, since plants are often subjected to multiple stresses. This should also be extended to the level of abiotic versus biotic stresses since many diseases are coupled with abiotic stresses at a certain stage of development of common bean. The available research on genomic, transcriptomic, and proteomic level on isolate stress-inductive factors should now be reunited in an attempt to elucidate the most complex phenomena involved in stress interactions. And, that should be extended to another level of complexity, which is establishing the interaction of both abiotic and biotic stress sources on common bean.

Regarding plant/pathogen interaction, until the moment the pathosystem *Phaseolus vulgaris*/ *Colletotrichum lindemuthianum* was only investigated in an incompatible interaction. However,

there are other combinations of genotype and pathogen races that lead to a compatible interaction and remain to be studied in order to compare these systems and understand which mechanisms are really responsible for the resistance.

Still, considering plant/pathogen interaction, in the past years, the LMD (laser micro-dissection) technology has been applied to study individual cells of plant-infected tissue and/or pathogen structures. This is because the way plant tissues were collected to do quantitative analyzes, as transcriptomic and proteomic, could generate a dilution of those cells in direct contact with the fungus into the whole tissue. This type of analysis allows a specific and localized evaluation. The LMD technique is based on the coordinated use of microscopy, laser and robotic, to localize, dissect, and capture cellular material [113]. This method has been important in selection and sampling of cells or cellular content in enough quantity and quality for DNA, RNA, protein, and metabolite analyzes, even in high throughput. Our group is employing this technology to study *P. vulgaris*/*C. lindemuthianum* interaction and *P. vulgaris*/mycorrhiza interaction under drought stress.

Looking for stress-resistance sources in other species and introgressing genes to common bean is another alternative for genomic improvement. A good example relies on the research that has been done for drought tolerance in common bean, based on interspecific crosses with other species of *Phaseolus*, such as tepary beans (*P. acutifolius*). They naturally span from the desert highlands of northwest Mexico to the southwest of the USA and thus they are good sources of drought, heat, and cold tolerance [114]. An interesting feature of tepary beans is their root system, which reveals extremely fine roots with rapid penetration in the soil with profuse branching, which enables quick access to limited soil water [115].

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# Stress Responsive Non-protein Coding RNAs

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## Abstract

Environmental or growth conditions are constantly influencing the development and maturation of organisms. To ensure precise control of gene expression under these normal as well as stress conditions, organisms developed very accurate molecular regulatory mechanisms at transcriptional and posttranscriptional levels. Key roles of small noncoding RNAs (miRNAs and siRNAs) in regulation of gene expression are no longer astonishing us nor are constantly emerging new classes of non-protein coding RNAs (tRNA-derived small RNAs, snoRNA-derived small RNAs, mRNA-derived small RNAs, and long non-coding RNAs). Although there is evidence of involvement of those various classes of noncoding RNAs in response to stress conditions, in most cases, we are still lacking basic information on their targets and interplay between different regulatory pathways. In this review, we focus on “canonical” and “noncanonical” noncoding RNAs and their involvement in abiotic stress responses.

**Keywords:** Noncoding RNAs, abiotic stress, crops, yeast

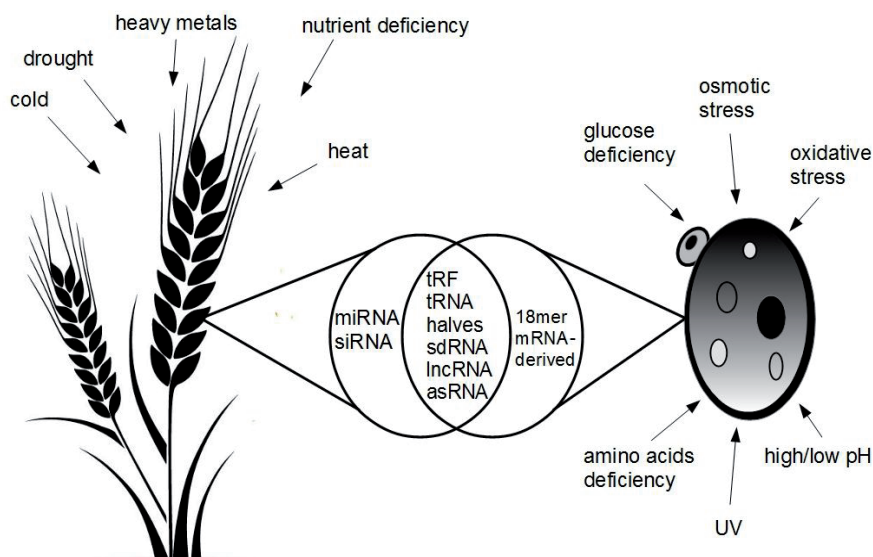
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## 1. Introduction

Biological organisms exposed to any external circumstances that exert a detrimental influence on them are capable of establishing mechanisms of protection and adaptation. To minimize stress influence, several different strategies can be applied, these are tolerance, resistance, and avoidance or ultimately escape. Because of their sedentary lifestyle, plants are restricted to tolerance, resistance, and avoidance mechanisms only, and thus require short-term strategies to quickly and efficiently readapt their metabolism [1]. Therefore, to ensure their survival under unfavorable conditions plants have established refined adjustments to stresses at all levels (anatomical, morphological, cellular, biochemical, and molecular) [2]. On the other hand, microorganisms, such as industrially important baking yeast, have specific and delicately

balanced internal conditions for optimal growth and function. Industrial applications expose yeast to multiple unfavorable environmental conditions, therefore accurate stress response is key for yeast cells. When environmental conditions change abruptly, yeast cells rapidly adjust its internal balance to that required for growth in the new conditions.

The physiological and biochemical changes in plants under stress conditions are a result of alteration in abundance of many transcripts and therefore proteins [3] pinpointing the role of TGS (transcriptional gene expression) and PTGS (post-transcriptional gene expression) in the adaptation to environmental changes. To achieve precise and timely regulation of stress-responsive gene networks in addition to regulatory DNA sequences and different types of proteins, plants, as well as many other organisms, including yeast, have employed the gene expression regulation by RNA molecules.



Differentially expressed RNAs are involved in regulation of plant and yeast metabolism pathways during development as well as stress conditions. Baking yeast are depleted of RNAi dependent molecules (miRNA and siRNA) that are present in plants.

**Figure 1.** Overview of different classes of ncRNAs associated with response to abiotic stress conditions in plants and yeast.

For many decades RNA was considered to be a passive intermediate in the flow of information from DNA to protein, apart from other well-known and described functions in translation (tRNAs, rRNAs) or splicing (UsnRNA). In 1998, its new role has been discovered and regulatory capacity of RNA molecules immensely broadened our understanding of gene expression processes. Moreover, new sequencing technologies and high-resolution microarray analysis

have revealed genome-wide pervasive transcription in many eukaryotes, generating a large number of RNAs of no coding capacity. Now, nearly 20 years after the first discovery of the RNA interference, we know that small non-coding RNAs not only regulate gene expression on post-transcriptional and transcriptional levels, but can also affect the organization and modification of chromatin. Moreover, they regulate growth and development of organisms, organ development, hormone signalling, and the defense against pathogens [4, 5]. They also function during environmental stress adaptation, providing precise regulation of gene expression.

Herein, we wish to describe the roles of non-protein coding RNA molecules during abiotic stresses in industrially important organisms, such as crops and yeasts. We wish to focus on “canonical” small RNAs, such as miRNAs and siRNAs, but also new classes such as mRNA-derived RNAs, tRNA-derived small RNAs, snoRNA-derived small RNAs (sdRNAs), mRNA-derived small RNAs, and long non-coding RNAs (lncRNAs) (Figure 1).

## 2. Canonical small RNAs

In plants, small RNAs exhibit unexpected complexity, but two main categories can be distinguished based on their biogenesis and function and these are miRNAs (microRNAs) and siRNAs (short interfering RNAs). Baking yeast lack these two categories of small RNAs since their biogenesis and functions involve RNAi machinery, which is absent in *S. cerevisiae*.

### 2.1. miRNAs

miRNAs are predominantly 21 nucleotides in length [6] and they are derived from 70–500 nucleotide long, imperfect, hairpin-like structured primary transcripts called pri-miRNAs by the activity of RNase III dicer-like (DCL) enzyme [7]. DCL1 processes primary miRNA transcript into an miRNA-miRNA\* duplex with 2nt overhangs at the 3' end [6]. With the help from hyponastic leaves (HYL1) and SERRATE proteins miRNA duplex is released and then Hua Enhancer 1 (HEN1) methylates 3' ends of the duplex and therefore stabilizes it by preventing uridylation and subsequent degradation. HASTY, the plant homolog of exportin 5, transports the miRNA duplex into the cytoplasm [8, 9]. The mature miRNA is loaded into RISC (RNA-induced silencing complex) and guides the translational inhibition or cleavage of target mRNAs through the action of AGO proteins in a sequence-specific manner [8–10].

To date, many stress-responsive miRNAs have been identified and the vast majority of their targets are transcriptional factors or proteins playing important roles not only in developmental and growth processes but also in biotic and abiotic stress responses [11]. There appears to exist common pathways of stress responses based on the induction/reduction of similar small noncoding RNA entities. It has been shown that different stress conditions trigger similar responses in various plant species; but on the other hand, the plant's response to similar stress condition could trigger the expression of different pools of miRNAs even in different varieties of the same plant species.

Drought and salinity are major external factors influencing crop productivity and seed quality worldwide. Understanding plant's responses to these stresses is therefore of high importance and one of the main research topics nowadays. Several studies showed differential expression profiles of many genes during water deficit conditions [12, 13]. Recent efforts have also led to the identification of many miRNAs altered in response to drought in many plant species such as cowpea [14], soybean [15], or *Triticum dicoccoides* [16]. Strikingly, the plant response varies in different species, depending on the habitat or plant family, resulting in majority of miRNAs having differential accumulation patterns; nevertheless, some miRNAs share common accumulation pattern as shown below. The differences observed are not only attributable to plant species or growth conditions but could also result from different genotypes in the same species. Soybean seedlings representing two different genotypes (drought-sensitive and tolerant) showed an increase in the level of five miRNAs (miR166-5p, miR169f-3p, miR1513c, miR397ab, and miR-seq13) [15]. In wild emmer wheat, 13 miRNAs were found to be differentially regulated upon drought (miR1867, miR896, miR398, miR528, miR474, miR396, miR894, miR156, miR1432, miR166, and miR171) [16]. Interestingly, some miRNAs share common accumulation pattern; for example, miR474 has also been shown to be upregulated in maize under water deficit [17]. Genome-wide approach allowed to identify 30 miRNA families significantly down- (16: miR156, miR159, miR168, miR170, miR171, miR172, miR319, miR396, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088, and miR1126) or upregulated (14: miR159, miR169, miR171, miR319, miR395, miR474, miR845, miR851, miR854, miR896, miR901, miR903, miR1026, and miR1125) under the drought stress in *Oryza sativa*; strikingly 9 miRNAs (miR156, miR168, miR170, miR171, miR172, miR319, miR396, miR397, and miR408) showed opposite expression patterns to previously described in drought-stressed *Arabidopsis* [18]. Some miRNA gene families were identified in both down- and upregulated groups (miR171, miR319, miR896). One miRNA that is consistently being upregulated during drought is miR393. The increase in the level of this microRNA has been observed for rice, *Arabidopsis*, *Medicago truncatula*, and *Phaseolus vulgaris* [19–23].

It is established that somewhere between 6% to 20% of worldwide cultivated arable land is affected by excessive concentrations of salt [24–26]. Some cellular and metabolic processes observed in plants during salt stress conditions (as a result of the limitation of the plant's ability to uptake water) are similar to those observed during drought [24]. Nevertheless, there are many genes and pathways that discriminate these two stress conditions [12, 28]. In salt-tolerant and salt-sensitive *Zea mays* lines, microarray hybridization experiments led to the identification of 98 salt-responsive miRNAs representing 27 families. Several microRNAs showed differential accumulation levels upon salinity in roots: members of miR156, miR164, miR167, and miR396 families were downregulated while miR162, miR168, miR395 and miR474 were upregulated [28]. In rice, other microRNAs appear to be involved in response to salt stress, these are miR169g, miR169n, miR169o, as well as miR393 [29, 30]. These microRNAs target the transcript of NF-YA gene encoding for a subunit A of a conserved transcription factor previously shown to be downregulated in drought-affected wheat [31]. Three microRNAs (miR414, miR164e, and miR408) have been established to be downregulated under salinity in rice [32]. Although, their target genes OsABP (ATP-binding protein), OsDBH (DEAD-box helicase), and OsDSHCT (DOB1/SK12/hely-like DEAD-box helicase), were for the first time

validated and upregulated in response to salt stress their functions still need to be experimentally confirmed.

Cold stress is one of the most severe abiotic stresses. The disorders triggered by low temperatures depend on the duration of stress condition and the rate of temperature decrease. Cold stress includes chilling (above 0°C) that inhibits water uptake and freezing (below 0°C) that induces cellular dehydration and thus osmotic stress and hyper accumulation of reactive oxygen species [33]. There are several factors (genes and transcription factors) playing important roles in cold stress acclimatization [10, 34–37]. Post-transcriptional mechanisms of cold acclimation include pre-mRNA processing, mRNA stability, and export from the nucleus. During the last few years, an emerging role of microRNAs in cold stress acclimatization and responses began to unveil. Cold, as well as ABA, dehydration, and salt stress upregulate the expression of miR393, miR397b, and miR402. In accordance with the upregulation of miR393 under cold stress, its target, putative E3 ubiquitin ligase SCF complex F-box protein, appears to be downregulated by cold stress in *Arabidopsis* [38]. Thus, during cold acclimation, the cleavage of E3 ubiquitin ligase mRNA would lead to diminished proteolysis of its targets (probably positive regulators of cold stress). Moreover, one of the targets of miR393 encodes an F-box protein (At4g03190) [39, 40], which is similar to glucose repression resistance 1 (GRR1), a yeast protein involved in glucose repression. Plants can use sugar status as a signal to modulate growth and development in response to abiotic stresses since it has been observed that sugar metabolism is affected by various abiotic stresses. It has been speculated therefore that miR393 could integrate sugar signaling with cold stress responses [41]. Moreover, in sugarcane, it has been observed that miR319 is differentially expressed during cold treatment for different periods of time, in both shoots and roots [42]. These data are in accordance with previous reports showing that miR319 was upregulated in response to cold stress in *Arabidopsis* [39, 40]. Genome-wide experiments in *Prunus persica* led to the identification of miR156, miR159, miR164, miR167, miR172, miR393, miR396, miR414, miR2275, and miR5021 as cold responsive [43, 44]. They are regulating genes involved in flower and leaf developmental processes. In rice, 18 miRNAs have been shown to respond rapidly to cold stress conditions (miR156, miR166, miR166m, miR167a,b,c, miR168b, miR169e, miR169f, miR169h, miR171a, miR535, miR319a,b, miR1884b, miR444a.1, miR1850, miR1868, miR1320, miR1435, and miR1876). Most of them are downregulated, which suggests that their targets are being upregulated in adaptation to cold stress. Interestingly, there were four miRNAs (miR1435, miR1876, miR1320, and miR1884) present in rice, but not *Arabidopsis*, in response to cold stress, which suggests a species-specific mechanisms [10]. miR171 is a large family consisting of 12 known members. In *Arabidopsis*, miR171a was induced 6 h after cold treatment [40]. On the other hand, miR171a in rice (rice and *Arabidopsis* share the same mature sequence) was significantly downregulated. Moreover, the expression levels of the other three miRNAs (e, f, i) were also decreased in rice in response to cold. In contrast, miR171 c, d, and h were induced after 6 h [10]. The difference in expression pattern as in the case of miR171 could be reflective of divergence in response of particular plant species to cold stress.

miRNAs also appear to play crucial roles in regulation of nutrient homeostasis. There are 14 mineral elements essential for plant growth and development. Phosphate (Pi) is one of the most prominent ones; it is involved in phosphorylation reactions, synthesis of nucleic acids,

membrane lipids, but also in energy delivery and often is a limiting nutrient for plant growth. The phosphate acquisition from soil particles requires the secretion of plant enzymes that release phosphate, then phosphate transport through Pht1 family of transporters (phosphate transporters protein family 1). Phosphate homeostasis in cells is regulated through the action of PHO1 and PHO2 proteins, transcription factors PHR1, WRK75, ZAT6, bHLH32, MYB62, in *Arabidopsis* and PTF1 in rice and microRNAs. MiR399, upregulated in low-phosphate stressed plants [45–47] targets two genes; a phosphate transporter PHO2 and a putative ubiquitin conjugating enzyme UBC24 transcript [6, 48] have been isolated from the phloem and their level increases in the phloem upon phosphate starvation, suggesting their role as phosphate starvation signals being translocated from shoots to roots where they promote phosphate uptake by downregulating PHO2 [49, 50]. Recently, miR-827-3p was shown to be upregulated in nitrogen and phosphorus deficiency conditions [51, 52]. In rice, it has been shown to target two genes encoding proteins containing SPX and MFS (major facilitator superfamily) domains. SPX-domain proteins are involved in Pi sensing and/or Pi transport [53]; whereas MFS-domain proteins are membrane proteins functioning in small molecule transport [54]. It has been suggested that SPX-MFS proteins might be involved in intracellular or intercellular Pi transport through regulating the expression of Pi transporter (PT) genes [55] and/or Pi storage or metabolism [56]. Similarly, in *Arabidopsis*, it targets the nitrogen/phosphate balance regulator Nitrogen Limitation Adaptation (NLA, AT1g02860) transcripts [57]. Overexpression of miR827 under Pi-starvation conditions causes the downregulation of NLA (together with downregulation of PHO2 through the action of miR399) leading to increased levels of phosphate transporter 1 (PHT1) and elevated activity of Pi uptake at the plasma membrane.

Sulfur (S) is another fundamental macronutrient necessary for proper growth and development. For plants, it is available from the soil in sulfate form, it is taken up by the roots, and distributed within the whole plant by sulfate transporters. It is found in amino acids, peptides and proteins, iron-sulfur clusters, and several co-factors, but also in an oxidized form in polysaccharides, lipids, and modified proteins [58, 59]. Sulfate deprivation induces the accumulation of miR395 that targets ATP sulfurylases (APSs) and sulfate transporter AST68 [6, 60], thus reducing their levels. In Brassica plants, deprived of sulfur, the level of miR395 increases in the root, stem, and leaf tissues, it is however strongest in the phloem [61, 62].

The regulation of copper (Cu) homeostasis is also managed through the action of microRNAs. This micronutrient is indispensable for photosynthesis and plant protection against reactive oxygen species [63, 64]. Under copper-deficient conditions, upregulation of miR398 leads to the downregulation of cytosol and chloroplastic Cu/Zn superoxidase dismutase transcripts level (CSD1 and CSD2) and copper chaperone for superoxide dismutase CCS1 [65, 67]. Cu/Zn SOD can be, therefore, replaced with FeSOD in chloroplasts [68]. The level of miR398 is elevated in Brassica under copper deprivation in leaf, stem, and root tissue but most importantly in the phloem [62], such as in the case of sulfur deprivation, which is in accordance with other research suggesting that the phloem has a distinct set of microRNAs from leaves and roots and responds specifically to stress conditions [63]. Several other microRNAs have also been recently linked with copper-deficient conditions (miR397, miR408, and miR857). They are predicted to target Cu-containing proteins (laccases and plantacyanin) [66].

Under high, heavy metal concentrations, plants are subjected to cation imbalances that lead to alterations in plant physiology and biochemistry. It is not surprising, therefore, that in response to these kind of stresses a set of microRNAs is differentially regulated. It has been observed in rice [68], *M. truncatula* [69], *Brassica napus* [70], and *Arabidopsis* [71]. In *Brassica*, several microRNAs have been linked with cadmium exposure (miR156, miR160, miR164b, miR171, miR393, miR394a,b,c and miR396a,) [70, 72]. In rice, assorted microRNAs have been upregulated in roots (miR601, miR602, and miR603) while some were downregulated in leaves (miR602 and miR606) or in roots (miR604) [68]. The levels of miR171, miR319, miR393, and miR529 were all heightened in response to mercury (Hg), cadmium (Cd), and aluminium (Al) exposure in *Medicago* [69].

## 2.2. siRNAs

siRNAs are derived from double-stranded RNAs that result from transcription of inverted-repeat sequences, natural cis-antisense transcript pairs, or by the action of RNA-dependent RNA polymerases (RDRs) that convert single-stranded RNA transcripts (e.g., virus replication) into double-stranded RNAs [8]. They also derive from heterochromatic regions and DNA repeats and mediate the silencing maintenance of the regions from which they originate [73]. These are then cleaved by dicer-like (DCL) enzymes to produce 21-24-nucleotide long species, the size depends on the specific catalytic activity of the respective DCL protein. Similarly to miRNAs, siRNAs are then loaded into Argonaute (AGO) protein-containing RISC that guides target regulation at the transcriptional level via the so called RNA-directed DNA methylation (RdDM) or at post-transcriptional level [8, 26, 74]. Several classes of siRNA can be distinguished, such as trans-acting siRNAs (tasiRNA), heterochromatic RNAs (hc-siRNA), and natural antisense siRNAs (nat-siRNA).

Tasi-RNAs are derived from specific genetic loci called TAS genes through a microRNA-dependent pathway [75, 76]. Cleaved TAS transcripts are converted into dsRNAs by RNA-dependent RNA polymerase 6 (RDR6), which are in turn cleaved into 21-nt siRNAs. Four families of TAS genes have been identified in *Arabidopsis*. TAS2 siRNAs target PPR mRNAs (pentatricopeptide repeat); TAS3 siRNAs target ARF2, 3 and 4 transcription factors; TAS4 siRNAs decrease the level of MYB transcription factors [78]. TAS1 and TAS2 are recognized by miR173, TAS3 by miR390 and TAS4 by miR828 [64]. TasiRNAs derived from TAS4 have been implicated in Pi deficiency conditions. They are directly involved in the biosynthesis of anthocyanins in response to low Pi [78]. A putative siRNA pta22 from *Pinus taeda* has been implicated in the response to pathogens, targeting transcripts of two genes encoding for disease resistance proteins [79]. The levels of TAS1, TAS2, and TAS3 tasiRNAs have been elevated in hypoxia-treated samples in *Arabidopsis* suggesting their role in stress responses [80]. These changes in tasiRNA levels have been proved to be correlated with the levels of TAS-targeting miRNAs (miR173 and miR390). The decrease in PPR transcripts level can be associated with the protection of mitochondria during hypoxia stress.

The usage of high-throughput sequencing methods allowed to identify thousands of genes in convergent overlapping pairs that can generate complementary transcripts in rice and *Arabidopsis* [41, 81, 82]. A natural cis-antisense transcript pair SRO5-P5CDH nat-siRNA plays



an important role in osmoprotection and oxidative stress management that results from high salinity [83]. P5CDH (pyrroline-5-carboxylate dehydrogenase) and SRO5 (similar to RCD One) are both involved in regulation of proline metabolism. The downregulation of P5CDH transcripts causes accumulation of proline, which is an important mechanism involved in the increased tolerance to salt stress conditions. But the decrease in the level of P5CDH causes an increase in a toxic metabolite P5C and reactive oxygen species that are detoxified by SRO5 proteins in the mitochondria [83].

There has also been a report showing changes in 4 siRNA levels in wheat seedlings under cold, heat, salinity, and dehydration stresses. Two of them (named 002061\_0636\_3054.1 and 005047\_0654\_1904.1) were downregulated in heat, salinity, and dehydration. The latter one was also responding to the cold stress by being upregulated. The level of another one (080621\_1340\_0098.1) was decreased under heat but upregulated under cold stress. The fourth one (997927\_0100\_2975.1) was not differentiating only under heat stress, but downregulated when plants were treated with salinity, cold, and dehydration conditions [84].

Heterochromatic 24-nt siRNAs are essential components of RdDM pathway [85]. The pathway has been established to protect plants from invasive nucleic acids, such as transposable elements and repetitive sequences, through DNA methylation and chromatin modifications [86–88]. It has been shown that 21-nt and 24-nt hcsiRNAs are the most abundant class (80%) of all small RNAs in plants [60, 89, 90]. The role of hcsiRNAs in abiotic stress responses is not well understood; although in the 1980s, McClintock has already suggested that all kinds of stresses could potentially reshape a plant genome via transpose activation [91]. Global analysis in plants such as *Arabidopsis* and rice suggests that the vast majority of transposons are inactive, methylated, and targeted by siRNAs [92]. Recently, few researches suggested that hcsiRNAs could play an important role in the adaptation to stress conditions. In *Arabidopsis* seedlings subjected to heat stress, ONSEN (copia-type retrotransposon) levels have been shown to increase in mutants affected in RdDM pathway [93]. After the recovery period, the level of ONSEN transcripts decreased gradually suggesting a role for hcsiRNAs. What is interesting is that the insertion of new ONSEN copies has been observed during flower development but before gametogenesis. Surprisingly, ONSEN insertions were also able to confer heat stress responsiveness to nearby genes. Together, all these data suggest that hcsiRNAs can contribute to control of transgenerational retrotransposition triggered by abiotic stresses and can participate in new regulatory networks established to protect plants against stress conditions. The detection of mPing transposition into a rice homologue of flowering time gene-CONSTANS in stressed cultivars supported this intriguing hypothesis. It turns out that stress-activated transposons could positively contribute to genome adaptation to growth in colder climates (reviewed in [1]). Moreover, stress-mediated induction was shown for Tos17 (rice), Tto1 (tobacco), Tnt1 (tobacco), and BARE-1 (barley) retrotransposons. Low temperature treatment was shown to decrease methylation and increase the excision rate of Tam3 transposon by binding its transposase to GCHCG sites immediately after DNA replication, thus preventing de novo sequence methylation (reviewed in [1]).



### 3. Non-canonical small RNAs

In plants, RNAi-dependent as well long ncRNAs-dependent mechanisms constitute the vast majority of stress response mechanisms. However, other non-protein coding RNAs are continuingly being discovered. At the same time, *S. cerevisiae* lacks the RNA interference pathway, and thus the ncRNA-based gene regulation is performed solely by the RNAi-independent mechanisms. Newly identified small non-coding RNAs other than miRNAs and siRNAs, their functions in gene regulation in yeast and plants, as well as possible mechanisms of action are discussed below.

#### 3.1. tRNA-derived small RNAs

In the expanding repertoire of small noncoding RNAs, tRNA-derived RNA fragments have been identified in all domains of life [reviewed in 94]. According to the nomenclature based on tRNA fragment size and the part of the tRNA molecule from which fragments are derived [95], tRNA fragments can be separated in two major classes: tRNA halves and smaller tRNA fragments (tRFs). In most of the cases reported till now, full-length tRNA levels do not decline significantly and tRNA fragment levels are consistently lower than those of full-length tRNAs, suggesting that only a small proportion of tRNAs is subjected to cleavage. This is in contrast to the complete depletion of tRNAs targeted by colicins in bacteria [96]. Recent evidences suggest that tRNA-derived fragments are not just a random degradation products but rather stable entities that may have major biological functions. The direct influence on protein biosynthesis has been revealed since: (i) tRF binds to 30S and inhibit translation in *Archaea* [97] and (ii) tRNA halves in human cell lines inhibit protein synthesis by displacing eIF4G/eIF4A from uncapped>capped RNAs [98].

So far, only few reports on plant tRNA-derived fragments are published. Such small RNAs have been observed under non-stress conditions in plants [99]; however, the basal levels of tRNA halves are low and often increase during stress conditions. In 2009, it has been reported that tRNA fragments are present abundantly under phosphate (Pi) deficiency in *Arabidopsis* [78]. In this study, a significantly higher amount (almost six-fold) of small RNAs derived from tRNAs was present in the roots than in the shoots. The accumulation of tRNA fragments in the -Pi roots was 1.4 times higher than that in the +Pi roots. When the origin of these RNAs was further analyzed, no correlation was observed between the abundance of small RNAs from specific tRNA species and their codon usage. What gained a special interest is the observation that a 19-nucleotide sequence processed from the 5' end of tRNA<sup>Gly</sup><sub>TCC</sub> represented over 80% of all tRNA-derived small RNAs in the roots and accounted for up to 18.44% and 27.70% of total sequence reads in the +Pi and -Pi root libraries, respectively, compared with only 1.00% to 1.79% in the shoot libraries. These results revealed a spatial and temporal expression pattern of small RNAs derived from the specific cleavage on tRNA molecules. The authors speculated that such differential accumulation of tRNA fragments between roots and shoots may represent the consequence of long-distance movement. Similar movement phenomenon of tRNA halves was observed in the phloem sap of *Curbita maxima* [100]. In this study, the presence of all tRNA anticodon families was inspected by northern blot hybridiza-

tion assays. In 12 out of 20 probed tRNA cases, stable processing products were detected in the phloem sap but not in leaf tissue extracts. The size of tRNA fragments ranged from 31 to ~60 nt and they derived from both 3' or 5' ends of particular tRNA molecule. The authors proposed that these phloem-delivered tRNA fragments may be a long-distance signal to coordinate the metabolic status between source and sink tissues.

A comprehensive expression profile of non-coding small RNAs was also performed in barley (*Hordeum vulgare* L.) under phosphorous-deficient and -sufficient conditions [101]. The deficiency in phosphorus decreases the plant growth and grain yields. Hackenberg et al., using high-throughput sequencing techniques, found that 56 out of the total 61 tRNAs were generating stable fragments in both P-deficient and P-sufficient shoots. Six tRNA-derived fragments were significantly upregulated, whereas four were significantly downregulated in P-deficient shoots. Notably, the read count of tRNA fragments was two-fold greater in P-deficient shoots than in P-sufficient shoots. The most abundant of these small RNAs was tRF derived from tRNA<sup>Gly</sup><sub>TCC</sub> (58.6% of the total tRFs in P-deficient shoots and 58.2% in P-sufficient shoots). tRNA<sup>Ala</sup><sub>AGC</sub>-derived sRNAs (previously reported as the most abundant in rice meristems) were the second most abundant species in P-sufficient shoots, but the third in P-deficient shoots. Such differential accumulation of tRFs between the two P treatments indicate that P has a great impact on the tRNA processing to small RNAs.

Deep sequencing technologies also provided evidence that novel subset of small RNAs are derived from the chloroplast genome (csRNAs) of Chinese cabbage [102]. The chloroplast small RNAs (csRNAs) included, among others, those that derive from tRNAs. Wang et al. found that the csRNAs derived from most of the chloroplast tRNA sequences constituted 5' parts of the molecules. Moreover, the first nucleotide of these csRNAs were predominantly located at the first nucleotide of the mature chloroplast tRNA sequences, revealing that the biogenesis of csRNA in tRNA molecules was specific to the 5' end. The tRNA-derived csRNAs only slightly declined in the heat-treated seedlings. However, the length of these csRNAs was related to heat stress response. In the heat-treated seedlings, the abundance of longer csRNAs (29–32 nt) decreased, but the of the shorter ones (16–25 nt) increased. csR-trnA-1 and csR-trnA-2 were the two most predominant tRNA-derived csRNA families and originated from chloroplast tRNA<sup>Ala</sup>. Such reduced abundance of tRNA-derived fragments 1 h after the heat treatment (the time period before the appearance of leaf etiolation) may suggest that the heat-responsive csRNAs play roles in the maintenance of subcellular structures and photosynthetic capacity of chloroplasts. The csRNAs derived from tRNAs may, therefore, play a role similar to the reported tRNA halves under various stress conditions.

In 2013, Loss-Morais et al. summarized all existing sequencing data aiming at the characterization of plant tRNA-derived fragments, their accumulation patterns under abiotic and biotic stresses, the identification of their putative targets, as well as possible association with Argonaute (AGO) proteins [103]. They have inspected 34 Arabidopsis deep sequencing libraries, including 25 AGO-IP libraries and found tRFs in the AGO1, 2, 4, and 7 IP libraries. Both, 5' and 3' tRFs were associated with AGO, mirroring previous results in mammalian systems [104–105]. Interestingly, tRFs from the central part of tRNAs were also detected, although 5' tRFs formed the most abundant class and showed the highest sequence diversity.

The AGO-associated 5' tRFs were predominantly 19-mers. Then, to investigate if the 5' tRFs associated with AGOs act in the RNAi pathway in plants, as has been suggested in animals [104], the authors searched for tRF targets in Arabidopsis and identified four possible target genes. They have found that drought conditions enhanced the expression of the four tRFs, including the 5'-tRF<sup>Gly</sup><sub>TCG</sub>, which was already known to be up-regulated in response to phosphate deprivation [78].

The existence of transgenerationally transmitted, heat-responsive tRNA-derived fragments in plants was revealed for the first time by Bilichak et al. in *Brassica rapa* [106]. In the Bilichak study, small RNA sequencing was performed to compare alterations in RNAs in somatic and reproductive tissues of *B. rapa* plants and in their progeny in response to heat treatment. The authors have demonstrated high tissue-specific alterations in the small RNA accumulation profiles in tissues that were not directly exposed to stress, namely, in the endosperm and pollen. Importantly, they have revealed that the progeny of stressed plants exhibited the highest fluctuations in the small RNA accumulation levels (tRNA fragments among them). It has been speculated that perturbations in the expression of small RNAs in somatic tissues caused by environmental fluctuations would eventually be imprinted in the transcriptome patterns in gametes and progeny.

The presence of tRNA-derived fragments has also been reported in industrially important budding yeast *S. cerevisiae* [107, 108]. Thompson et al. were the first to demonstrate (already in 2008) that *S. cerevisiae* contain a small RNA population consisting primarily of tRNA halves and rRNA fragments [106]. tRNA fragmentation to stable shorter pieces occur in yeast cells in the absence and in the presence of stress conditions. However, the accumulation of tRNA fragments in yeast was most pronounced during oxidative stress conditions, especially during entry into stationary growth phase. Both 5' and 3' fragments of tRNAs were detectable suggesting the occurrence of endonucleolytic cleavage. The nuclease involved in this cleavage was characterized one year later [109]. It has been shown in yeast that tRNAs are cleaved by RNase T2 family member Rny1p, which is released from the vacuole into the cytosol during oxidative stress. Rny1p modulates yeast cell survival during oxidative stress independent of its catalytic ability. This suggests that upon release to the cytosol, Rny1p promotes cell death by direct interactions with downstream components.

The possible function of tRNA fragments in yeast has been described in 2012 [108]. Although RNA interference (RNAi) is conserved in diverse eukaryotic species, including budding yeast *Saccharomyces castellii* and *Candida albicans* [110], it has been lost in *S. cerevisiae*. Thus, this organism provides an ideal system for studying the RNAi-independent mechanisms of tRF-based gene expression regulation. The potential novel function of tRNA fragments is supposed to appear as a response to specific environmental conditions and includes tRNA processing and association with the ribosomes. As a result of high throughput sequencing of ribosome-associated small RNAs, it appeared that one of the most abundant classes of processed RNAs were tRNAs. In addition to previously reported cleavage in the anticodon loop in yeast tRNAs [107], other breakage points were also detected (e.g., in the D- and T-loop regions), reminiscent to those observed previously in higher eukaryotes [111]. Moreover, an obvious differential stability of tRNA halves was recognized. Northern blot analysis confirmed the presence of two

stable processing products derived from tRNA<sup>His</sup> and revealed that cleavage is stress-dependent. Similar to previous findings tRNA processing was mainly detected during amino acid and sugar starvation conditions. On the contrary, experimental results obtained for tRNA<sup>Ser</sup> suggested that only the 3' part of this tRNA is stable. The association of tRNA fragments with yeast ribosomes suggest its potential regulatory function in protein biosynthesis, as already reported for *Archea* [97] and humans [98].

### 3.2. snoRNA-derived small RNAs

Small nucleolar RNAs are a highly evolutionarily conserved class of RNAs, which are present throughout the eukaryotes and whose origin lies in the Archaea. There are two classes of snoRNAs (C/D and H/ACA box) that function as ribonucleoprotein (RNP) complexes to guide enzymatic modification of target RNAs at sites determined by RNA:RNA antisense interactions. Generally, C/D box snoRNAs are ~70–120 nucleotides (nt) long and guide the methylation of target RNAs, while H/ACA box snoRNAs are ~100–200 nt long and guide the pseudouridulation of their targets. These RNAs were initially discovered in the nucleolus and thought to exclusively target ribosomal RNAs inside this sub-nuclear compartment. However, numerous snoRNAs do not possess target RNAs—therefore, they are called “orphan snoRNAs”.

Small nucleolar RNAs are localized in the nucleoli and Cajal bodies in eukaryotic cells. Components of the RNA silencing pathway associate with these structures, and two recent reports have revealed that a human and a protozoan snoRNA can be processed into microRNA-like RNAs [112–113]. By systematic analyses of deep-sequencing libraries from diverse eukaryotic organisms, Taft et al. revealed that small RNAs with evolutionary conservation of size and position are derived from the vast majority of snoRNA loci in animals (human, mouse, chicken, fruit fly), *Arabidopsis*, and fission yeast [114]. These small RNAs derived from snoRNAs (sdRNAs) in *Arabidopsis* were strongly associated with AGO7 proteins. *Arabidopsis* Argonaute proteins preferentially load small RNAs with specific 5' nucleotides and AGO7 is selective for 5' uracil (U) and 5' adenine (A). Intriguingly, H/ACA sdRNAs are dominantly 5'A, while C/D box are dominantly 5'U. Therefore, it is likely that sdRNAs play roles in the regulation of gene expression and transcriptional silencing. In particular, the fact that both miRNAs and sdRNAs are 5'U biased strengthens the link between them and suggests that some snoRNAs, including “orphan” snoRNAs whose targets are unknown, may function solely as intermediates in the sdRNA pathway.

However, snoRNA-mediated change in gene expression should be treated not as an example but as an addition to siRNA-based mechanisms. The reason for this statement is that canonical snoRNAs interact with their target RNAs through their 10–20 nt long antisense-box elements. In contrast, snoRNA-derived small RNAs can interact with other sequence elements with their targets, as they form a different ribonucleoprotein complexes.

The presence of snoRNA processing products was experimentally verified by high throughput sequencing, as well as northern blot hybridization in *S. cerevisiae* [108]. The results confirmed the presence of a shortened version of both types of snoRNAs. The processing events were most prominent under most tested yeast growth conditions, which included UV radiation,

anaerobic growth, high or low pH treatment, amino acid starvation and sugar starvation. Like in the case of tRNA-derived fragments, the association of sdRNAs with the ribosomes implicates their possible regulatory role in protein biosynthesis in *S. cerevisiae*. In this organism, RNAi silencing pathway has been lost during evolution, therefore the mechanism of sdRNAs action in *S. cerevisiae* is likely to be different from microRNA-like sdRNA action in plants.

Despite the differences in possible mechanism of action of sdRNAs, for both plants and yeast, the presence of small regulatory RNAs in the nucleolus allows us to speculate that the nucleolus is involved in the regulation of expression, possibly in response to cellular conditions.

### 3.3. mRNA-derived RNAs

Small mRNA fragments have been reported in diverse eukaryotes and a portion of them is stress-related [reviewed in 115]. A part of them is annotated as antisense RNAs (asRNAs), overlapping protein coding transcripts on the opposite strand. Some asRNAs were found to repress sense RNAs and lead to inverse expression between asRNAs and their corresponding sense RNAs in respect to growth phases, stress conditions, or environmental changes. Therefore, the corresponding protein-coding transcripts (mRNAs) represent a mixture of cell-cycle factors, chromatin remodellers, and metabolism related proteins. It has been shown recently that about one-fifth of the ORF genes in *S. cerevisiae* are coupling with asRNAs; however, the ratio of genes coupling with asRNAs shows a negative association with gene regulatory complexity [116]. This observation supports previous hypothesis that some asRNAs belong simply to a transcriptional noise. Nevertheless, asRNAs evolve more slowly when their sense genes are under more complex regulation. Older genes coupling with asRNAs are more likely to demonstrate inverse expression, reflecting the role of these asRNAs as repressors, especially under heat stress stimuli.

Recent tiling assays in plants (mostly in Arabidopsis) also revealed the majority of stress-responsive novel antisense transcripts [117]. Despite a linear correlation between the expression ratios of selected sense and antisense transcripts, biological functions of most antisense RNAs remains unclear. In plants, it has been postulated that antisense transcripts might have the potential to produce endogenous siRNAs (nat-siRNAs) as mentioned in Paragraph 2.2. Recent genome-wide analysis reported an accumulation of sRNAs in their overlapping region, suggesting the occurrence of an RNA interference event [82]. However, the biological processes of generating nat-siRNAs is not completely understood at this time.

The power of mRNA-derived ncRNAs for rapid global translation attenuator in stress was recently demonstrated in *S. cerevisiae* [118]. mRNA exon-derived 18-residue-long ncRNA (picked up in previous genomic screen for ribosome-bound small RNAs in *S. cerevisiae*) has been functionally characterized [108]. This ribosome-bound ncRNA, originating as a short sense fragment of TRM10 open reading frame, is needed for the rapid shutdown of global translation and is capable of adjusting translation rates by interacting with polysomes under hyperosmotic growth conditions. Therefore, the existence of a largely unexplored mechanism of translation control has been clearly demonstrated.

#### 4. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are a wide group of molecules identified in yeast, plants, and mammals. In general, lncRNAs can be defined as polyadenylated or nonpolyadenylated, more than 200 nt long transcripts with low protein coding potential (coding for less than 100 amino acids). Acceleration in transcriptome research, achieved thanks to the development of high-throughput technologies such as microarrays or next-generation sequencing methods, allow us now to fully appreciate complicated interactions that lead to precise gene expression regulation. It turned out that apart from genes encoding proteins, transposons, genes for housekeeping RNAs (like ribosomal RNAs), intergenic regions, which lie between protein-coding sequences, are also being expressed. Intriguingly, similarly to mRNA, those transcripts, namely lncRNAs, are capped at the 5' end and many of them are targets of the splicing process [119]. However, in contrast to mRNA, their expression level is very low and they do not have long open reading frames, which are evolutionary conserved [120]. lncRNA also differ from protein-coding transcripts in ribosome occupancy [121]. Large-scale genomics projects, such as Encyclopedia of DNA Elements (ENCODE), proved that lncRNAs are not only transcriptome noise, but indeed transcripts with biological functions. As a result of the ENCODE project, it has been shown that 75% of human genome is transcribed and about 80% of those RNA molecules have some biochemical function [122]. Most of the intergenic regions of Arabidopsis, rice or corn are shown to be transcribed as well as human and constitute a source of lncRNAs that are polyadenylated. Such polyadenylated stable lncRNAs are transcribed by RNA polymerase II and can be divided into four groups based on their genomic origin and relationship with adjacent protein-coding genes:

1. intergenic lncRNAs (lincRNAs) that are transcribed from sequences between two genes
2. intronic ncRNAs (incRNAs) that overlap with intronic sequences within another transcript
3. natural antisense transcripts (NATs) derived from complementary DNA strand of their associated genes
4. sense lncRNAs overlapping with one or more exon sequences of the transcript on the same strand.

Recently in Arabidopsis and rice [123, 124], another category of lncRNAs has been described — these are nonpolyadenylated transcripts, 50-300 nt in length with low coding potential, but without any sequence similarity to known ncRNAs. This novel group is referred to as intermediate-sized ncRNAs (im-ncRNAs).

Despite the effort of 20 years of investigation [125], the elusive role of lncRNAs is still not fully described nor understood. Thus far functions of only few such molecules are characterized. We know that lncRNAs are engaged mainly in transcriptional gene expression regulation by acting as scaffolds for transcriptional factors and genetic modifiers, molecular signals, decoys or guides. Moreover lncRNAs can also encode for miRNA and target specific mRNAs for decay or function as miRNA sponges. Most studies has been performed on animal systems, but

although research on plants are limited, the emerging picture is that the regulatory functions of plant lncRNAs are similar to animal ones [126]. Till today, almost 40,000 putative lncRNAs have been identified in *A. thaliana* [127] and thousands of them in *Oryza sativa* [128], *Zea mays* [129], *Medicago truncatula* [130], *Populus trichocarpa* [131], and other plant species [132, 133]. With rapid development of bioinformatics tools and transcriptome analysis methodologies, genome-wide identifications of plant lncRNAs have been conducted. In maize implementation of SVM tools (support vector machines), together with Python pipeline on cDNA dataset resulted in the identification of 2,492 potential ncRNAs, which represent 13.3% of initial sequences. In total, 237 ncRNAs were classified as shRNA precursors, and 1,225 as siRNA precursors, which constituted 59.4% of predicted ncRNAs particles. The remaining 1,011 was considered to be potential long non-coding transcripts [134]. Recently, new gold standard to study the complexity of eukaryotic transcriptomes emerged — the RNA-sequencing technology (RNA-seq). It allows an accurate quantification of expression levels of transcripts and also reveals transcripts that are missing or incomplete from the reference genome. Computational prediction based on RNA-seq data from rice anthers, pistils, seeds, and shoots, together with 40 available rice RNA-seq libraries led to the identification of 2,224 reliably expressed lncRNAs, including 1,624 lincRNAs and 600 long non-coding natural antisense transcripts (NATs). Further verification of rice insertional mutants allowed to set a pool of lncRNAs that are preferentially expressed at the reproductive stage. Several lncRNAs were identified as competing endogenous RNAs (ceRNAs), which sequester miR160 or miR164 in a type of target mimicry [135].

Another feature that complicates the retrieval of true lncRNAs is their weak sequence conservation. It is estimated that only from 2% to 5.5% of lncRNAs are conserved in their primary sequence and only some of them may be associated with short conserved elements. Most likely, it is a result of rapid evolution — lncRNAs are frequent targets of positive selection [136]. Some lncRNAs and their target genes can be distinguished by their conserved synteny across species — those lncRNAs play roles in cis-functions [136]. Other lncRNAs may be recognized by conserved secondary structures, which allow them to interact with RNA-binding proteins [124]. Genome-wide analyses carried out so far determined that expression of different groups of lncRNAs is highly tissue-specific and many of them are responsive to biotic and abiotic stress conditions.

In conjunction with the climatic changes, drought is the condition that has been recently extensively studied, and thus many drought-responsive lncRNAs were identified. In *Populus trichocarpa*, a model tree species, RNA-seq experiments conducted on control- and drought-treated plants revealed 504 drought-responsive lincRNAs and allowed for basic annotation set of 2,542 of them. Mutual interaction of miRNA and lncRNAs was also reported; a total of 30 miRNAs were predicted to target the sense strand of lincRNAs, 21 were found to target the antisense strand, and 20 target mimicry events was predicted of known *Populus* miRNA [130]. A potential new model organism of the family Poaceae, *Foxtail millet*, was also subjected to water deficient conditions. Deep transcriptome sequencing revealed 585 lncRNAs responding to PEG-induced drought stress. Those stress conditions induced the expression of 17 lincRNAs and 2 NATs at different expression levels. Qi et al. [138] identified one lncRNA, whose



sequence was shared with its counterpart in sorghum. In maize, one of most important crop species, genome-wide identification of differentially expressed lncRNAs during drought conditions led to the identification of 567 upregulated and 97 downregulated lncRNAs, among them 538 particles were considered to be novel. Moreover, 8 lncRNAs molecules were homologous to the miRNA precursors, 62 were classified as both shRNA and siRNA precursors, and 279 were classified as siRNA precursors [139].

In the best known model plant, *Arabidopsis thaliana*, genome-wide characterization of lncRNAs was performed as well. A correlated expression of lncRNAs with its epigenetic and structural features in response to four stresses (heat, cold, drought, and salt) has been described [140]. The authors identified 245 polyadenylated and 58 nonpolyadenylated lncRNAs that are differentially expressed under stress stimuli, and most of the selected candidates were further validated by qRT-PCR. From experiments on *Arabidopsis* came best studied cases of plant lncRNAs functions such as: *COLDIAIR*, *COOLAIR*, *At4IPS1*, *npc48*, and *npc536* [141–145].

One of best described mechanism of lncRNAs action is lncRNA transcript IPS1 (Induced by Phosphate Starvation 1). IPS1 can interact with miRNA as a competitor and function as miRNA target mimics, which resembles the miRNA sponges from animal systems. Maintaining the phosphate balance is a complicated mechanism in plants, regulated, among others, by miR399 as described in Paragraph 2.1. Low activity of PHO2, ruled by mRNA cleavage mediated by miR399, causes the elevation of phosphate uptake by increasing the expression of two root phosphate transporters. Phosphate starvation also increases the level of IPS1 transcript that has a 23-nt conserved domain, partially complementary to miR399 with 3-nt mismatch overlapping with the miR399-mediated cleavage site. As a non-cleavable product, IPS1 competes with PHO2 and can therefore weaken the miR399-mediated repression of PHO2 [142]. The miRNA sponge strategy is used in the therapy of human diseases and similar processes in plants (target mimic) and can be a very useful tool in plant research as well as in agricultural applications. As mentioned before, in *Arabidopsis* to date about 20 putative target mimicry events were predicted, which suggest the potential role of this mechanism in other pathways than the maintenance of phosphate homeostasis [146].

Another model organism, *Saccharomyces cerevisiae*, allows researchers to define and clarify a large number of new and unexpected roles of lncRNAs, such as promoting the timing of gene expression [147], cell cycle regulation during stress conditions [148], or local reduction of histone density and chromatin remodeling in response to glucose starvation [149]. Upon osmotic stress in yeast, hundreds of stress-responsive genes are induced by the stress-activated protein kinase (SAPK) p38/Hog1. Whole-genome tiling arrays were used to identify a set of Hog1-induced lncRNAs. One of the genes expressing a Hog1-dependent lncRNA in antisense orientation is *CDC28*, the cyclin-dependent kinase 1 (CDK1) that controls the cell cycle in yeast. *Cdc28* lncRNA mediates the establishment of gene looping and the relocalization of Hog1 and RSC from the 3' UTR to the +1 nucleosome to induce *CDC28* expression. The increase in expression level of *Cdc28* makes cells able to re-enter the cell cycle more efficiently after stress conditions occur. This may represent a more general mechanism to prime the expression of genes needed after stresses [148].



## 5. Conclusions

The rapidly growing human population is constantly stressing agro-ecosystems. In 2007, a FAO (Food and Agriculture Organization of the United Nations) report stated that only about 3.5% of the global agricultural area is not affected by any of the environmental stresses. Since recent advances in biotechnology and molecular biology have dramatically changed our understanding of gene expression regulation and responses of plants to abiotic stresses, we are now armed in new tools that could help us fight various stress conditions and improve the growth and yield of crops and other industrially important species. Our journey in the small RNA world has begun in the past century, in the 90s, with the discovery of RNA interference pathway, followed by siRNAs and their role in DNA methylation and chromatin modifications. Consequently, new and relatively surprising small RNA groups (tRNA-derived small RNAs, snoRNA-derived small RNAs, mRNA-derived small RNAs) have been discovered and slowly but surely their role in cells and in the adaptation to various environmental conditions is being established. Concurrently, thousands of lncRNAs have already been identified and thousands of them are still waiting to be discovered in different organisms, tissues, or in response to different stimuli. With the advent of new high-throughput techniques, it became easier and much faster to collect a vast amount of different types of molecular data. The analysis of such a high amount of data and understanding of mutual correlations and relationships between genes, their roles, and small RNAs is now a challenge that researchers and scientists must face. Nevertheless, we believe that by combining new methodologies with the help of bioinformatic approaches and wet lab experiments, we can shed light on a genomic "dark matter" and start to appreciate important physiological roles of various types of non-protein coding RNAs, as well as their potential applications.

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## **Abiotic and Biotic Stress Tolerance: Metabolic Insights**

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## Secondary Metabolism in *Amaranthus* spp. — A Genomic Approach to Understand Its Diversity and Responsiveness to Stress in Marginally Studied Crops with High Agronomic Potential

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Additional information is available at the end of the chapter

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### Abstract

Cultivated amaranths are crops with an unrealized agronomical potential despite their high nutritional value and nutraceutical properties of their seeds and/or leaves. They tolerate growing conditions unsuitable for cereals, and are tolerant to biotic aggressors. Several *Amaranthus* species are abundant sources of secondary metabolites, mostly phenylpropanoids, predominantly in seeds and leaves, many of which may confer health benefits associated with their antioxidant properties. They could also act as defensive compounds against predators or pathogens. Recent biochemical and molecular approaches partly defined the mechanisms responsible for grain amaranth's tolerance against biotic stress. However, the role played by secondary metabolites in (a)biotic stress amelioration in amaranth is practically unknown. Our group has identified several genes coding for enzymes involved in secondary metabolism pathways in *A. hypochondriacus*, in addition to related regulatory transcription factors. More than 50% of these genes involve the phenylpropanoid pathway. In this chapter, the role played by this pathway in (a)biotic stress amelioration in plants will be briefly reviewed, followed by an examination of its involvement in the conferral of nutraceutical properties to amaranth plants. A description of the progress obtained so far regarding the characterization of phenylpropanoid genes in grain amaranth will close this chapter.

**Keywords:** (a)biotic stress, grain amaranth, phenylpropanoids

## 1. Introduction

Species belonging to the *Amaranthaceae* compose a diverse and interesting family of plants. They can develop in highly contrasting habitats, from arid and semi-arid zones, where they can survive in sandy alkaline and/ or serpentine soils, to disturbed tropical forests. A minority are found in aquatic, semi-aquatic or marine environments. Their high affinity to saline conditions stems, in part, from the weedy nature of most of the species that constitute the *Alternanthera*, *Amaranthus*, *Celosia*, *Chamissoa*, *Froelichia*, *Gomphrena*, *Guilleminea*, and *Iresine* genera. These plants are widely distributed in tropical and subtropical zones due to their ability to colonize or invade diverse habitats [1].

The *Amaranthus* genus is highly diverse, including approximately 70 species. A fraction of these may grow in saline soils; in this regard, *A. greggii* is considered to be a marker of saliferous soils. This genus includes grain-producing species, the most important being *A. cruentus*, *A. caudatus*, and *A. hypochondriacus*. Grain amaranths are valued for the high protein content and multiple nutraceutical properties of their seeds [2,3]. Moreover, they possess an inherent tolerance to high temperatures and drought, traits which have been associated with their C4 physiology, indeterminate flowering habit and superior water use efficiency due to their ability to grow long tap roots and develop an extensive lateral root system [4–6]. In concordance with related species within the *Amaranthaceae*, grain amaranths can tolerate poor and saline soils conditions and erratic rains unsuitable for the cultivation of other grain crops. Besides, they are not particularly susceptible to major diseases or insect pests [5–8]. Their high tolerance to severe defoliation, produced by mechanical means [9,10] or by insect folivory [6,11], is believed to contribute to this trait. In addition, they can readily respond to chemical elicitors of defense responses, such as jasmonic acid (JA) [12–14] or benzothiadiazole (BTH) [15], to increase their resistance against highly damaging phloem-feeding insect pests, such as the tarnished bug *Lygus lineolaris*, or against potentially lethal pathogenic bacteria. However, the contribution of secondary metabolites to (a)biotic stress tolerance in grain amaranth has been barely explored.

In this chapter, we shall first review the role played by phenylpropanoid pathway in the amelioration of both biotic and abiotic stress in plants. Then, we shall proceed to describe the state of the art with regard to the phenylpropanoid pathway in *Amaranthus*, mostly in relation to stress, but also in relation to their role in the biosynthesis of compounds with proposed nutraceutical properties. Finally, a thorough description of the progress we have achieved so far with respect to the genomic characterization of this particular secondary metabolic pathway in grain amaranth, mostly in terms of stress tolerance and/ or resistance will be made. The information gathered as a result of these efforts will expand the knowledge, perhaps into unknown territory, about the chemical diversity in plants, particularly in grain amaranth and related species, many of which can thrive in extreme habitats.

## 2. Reactive Oxygen Species (ROS)

### 2.1. Brief description of their ubiquitous and malignant role in plant stress and the antioxidant defense mechanisms induced for their control — What is known regarding amelioration of ROS-related damage in amaranth plants during stress?

The abundance of reactive oxygen species (ROS) tends to increase in the tissues of plants exposed to diverse environmental challenges including contact with heavy metal contaminants in soil, water or salinity stress, which is often accompanied by high light and temperature, and nutrient deficiency [16]. Most stress conditions in plants cause an accumulation of ROS, such as superoxide ion, hydrogen peroxide, oxygen-containing radicals, and others. These chemical entities can produce extensive oxidative damage in the apoplastic compartment and may also harm cellular membranes by lipid peroxidation. Additionally, they can have an impact on ion homeostasis mechanisms, which are crucial for many stress tolerance mechanisms, by interfering with ion fluxes [17]. ROS detoxification frequently involves the combined action of both antioxidant metabolites such as ascorbate, glutathione, tocopherols, and ROS-detoxifying enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) [18,19]. In this sense, overproduction of antioxidants in response to drought-induced oxidative stress has been frequently found to be associated with the drought stress tolerance of different plant species [20,21]. Also, enhanced drought tolerance has been generated in several different transgenic plants transformed with genes encoding diverse types of antioxidant-related enzymes or metabolites (e.g., SOD, APX, monodehydroascorbate reductase, and tocopherol cyclase, a key enzyme of tocopherol biosynthesis, [22]). Regarding grain amaranth, a series of proteomic studies performed in plants exposed to drought and saline stress detected the accumulation of various antioxidant enzymes, similar to those mentioned above [23,24]. In addition, the participation of antioxidant genes, such as *AhCAT*, *AhAPX*, and *AhSOD*, was implied by findings showing their induced-up-regulation in grain amaranth plants primed to resist infection by pathogenic bacteria [15]. However, a further characterization of the additional six *CAT*, four *APX*, and three *SOD* genes identified in grain amaranth [12] remains to be performed.

Glycine betaine (GB) is a quaternary ammonium compound that acts as an osmolyte with protective functions in plants subjected to the osmotic stress normally produced under drought, high temperature and/ or excessive salinity conditions. GB accumulation in the cytoplasm reduces ion toxicity, and ameliorates the highly damaging effects caused by the usually simultaneous presence of dehydration, salinity, and extreme temperature stresses. The protective effect is proposed to be exerted by the stabilization of macromolecular structures, and/ or by the protection of chloroplasts, particularly the photosystem II complex. The latter is believed to involve the thermodynamic stabilization of the indirect interaction of extrinsic photosystem II complex proteins with membrane phosphatidylcholine moieties [25]. GB is synthesized by the two-step oxidation of choline. The first step is catalyzed by choline monooxygenase, followed by the action of betaine aldehyde dehydrogenase. Both genes have

been described in different amaranth species [12,26,27], whereas the presence of GB has been reported in all Amaranthaceae species examined so far, with the exception of *Chenopodium quinoa* and *Noaea mucronata*. However, the GB concentration needed to exert a protective effect in plants of this family has been found to vary widely in a species-specific manner [25]. On the other hand, GB-accumulating transgenic plants usually show a significant improvement in their tolerance to different abiotic stress conditions [28].

## 2.2. Betacyanins in amaranth: More than pigments?

Betalains are water-soluble, nitrogen-containing pigments that are found only in one group of angiosperms, the Caryophyllales. For reasons that remain unresolved, they have never been found jointly with anthocyanins in the same plant [29,30]. This particular trait has been employed for chemo-taxonomical purposes. These pigments can be divided into the red-violet betacyanins and the yellow betaxanthins. Both are immonium conjugates of betalamic acid covalently bonded with cyclo-dihydroxyphenylalanine (cDOPA) glucosides, which can undergo further acylations [31–33]. These pigments are possibly needed for the optical attraction of pollinators and seed dispersers. Regarding stress, a protective role against accumulating ROS has been inferred from a number of studies [34–36] whereas the betacyanin amaranthine has also been proposed to exert protective effects against photo-oxidative damage in *A. tricolor* [36].

Recently, an analysis of key genes/ enzymes of the betacyanin biosynthetic pathway in *A. hypochondriacus* was performed. The study included genes coding for cyclo-DOPA glycosyl-transferase (*AhcDOPA5-GT*), two 4, 5-DOPA-extradiol-dioxygenase isoforms (*AhDODA-1* and *AhDODA-2*, respectively), a betanidin 5-glycosyl transferase (*AhB5-GT*), and an ortholog of the cytochrome P-450 R gene (*CYP76AD1*). The expression pattern of these genes, together with DOPA oxidase tyrosinase assays, was determined in both green and red tissues. The results obtained suggested that two apparently independent glycosylation pathways leading to betanins could be operating in *A. hypochondriacus* to synthesize amaranthine. In addition, these genes/ enzymes were shown to be induced differentially in a tissue-specific and genotype-specific manner in response to different stimuli, including water- and salt-stress and insect herbivory. The results obtained from the abiotic stress assays suggested that genes other than those examined in this study were probably contributing significantly to pigment content in tissues of stressed *A. hypochondriacus* plants. They also offered the possibility that these genes, due to their high induction by insect herbivory, particularly in acyanic plants, could function in defense responses against chewing insect pests by still undetermined mechanisms [37].

In addition to pigments, diverse phytochemical studies have shown that amaranth plants are capable of synthesizing a notable diversity of secondary metabolites [3,38,39]. Although many of these compounds are not considered to be essential for the primary needs of the plant, they are certainly required for survival in and/ or adaptation to challenging environmental conditions. Many of these compounds may be employed in amaranth and other plants as signaling compounds, in defense and/ or for communication with other organisms, such as pollinators [40–44].

The most commonly found secondary metabolite families found in amaranth and related species are phenylpropanoids, including flavonoids, phenolic acids, and their related amides, followed by alkaloids and terpenoids. From an anthropocentric perspective, some of these chemicals, including betacyanins, flavonoids polyphenols, and phenolic acids, are responsible for conferring amaranth and quinoa tissues with the bioactive antioxidant activity associated with their well-documented health benefit effects [3,38,45–49].

### 3. Phenylpropanoid secondary metabolites

Plants have accumulated a great diversity of phenolic compounds as a result of their long process of evolutionary adaptation. Approximately 40% of these compounds are derived from the highly diverse phenylpropanoid metabolism. The phenylpropanoid compounds constitute a highly diverse assortment of phenylalanine-derived secondary metabolites. These include flavonoids, which are generally sub classified into the anthocyanins, proanthocyanidins, flavonols, isoflavonoids, phlobaphenes, flavanones, and flavones subgroups, which are found in the majority of higher plants, in addition to the aurone subgroup, which is widespread, but not ubiquitous. Also included are monolignols, lignans, coumarins, phenolic acids, quinines, stilbenoids, and xanthones. Other phenolics include alkylmethoxyphenols, alkylphenols, curcuminoids, furacoumarins, hydroxybenzaldehydes, hydroxybenzoketones, hydroxycinnamaldehydes, hydroxycoumarins, hydroxyphenylpropenes, methoxyphenols, naphthoquinones, phenolic terpenes, and tyrosols.

Most of the flowers and fruits pigments employed for pollinator attraction and seed dispersal are water-soluble anthocyanins [50]. Proanthocyanidins, or condensed tannins, are colorless flavonoid polymers produced by the condensation of flavan-3-ol units [51]. Similarly colorless are the abundant flavonols which are usually found in the form of mono-, di-, or triglycosides [52]. Isoflavonoids and phlobaphenes are groups of flavonoids characterized by being predominantly found in the Papilionoideae family [53] or by their red pigmentation which results from the polymerization of flavan-4-ols [54], respectively. Based on their carbon skeleton, the ubiquitous phenolic acids can belong to the hydroxycinnamic acid type (chlorogenic, ferulic, rosmarinic, and sinapic acids) or to the hydroxybenzoic acid type (p-hydroxybenzoic, vanillic, and protocatechuic acids). Finally, the stilbenes represent a small family of phenylpropanoid metabolites dispersed in over 70 unrelated plant species [55–57]. Interestingly, the latter compounds are induced in response to several biotic and abiotic stimuli or by functionally related elicitors, such as methyl jasmonate (MeJA), and ethylene. Flavonoids are also involved in the regulation of auxin transport [58–60] and participate in the chemical dialog established between the plant roots and nitrogen-fixing bacteria and in signaling pathways designed to modulate ROS levels in plant tissues [61,62]. These compounds are also determining factors of male fertility and precursors for the synthesis of lignin [63–65]. The latter is an aromatic heteropolymer that confers mechanical strength to the cell wall and rigidity to plant stems, whose synthesis involves the assembly of p-coumaryl, coniferyl, and sinapyl alcohol monolignols. Lignin is also a waterproof insulator for cell walls and, as such, facilitates

the transport and assimilation of water through the vascular system. It also provides protection against wounding, UV light, and pathogen attack [66].

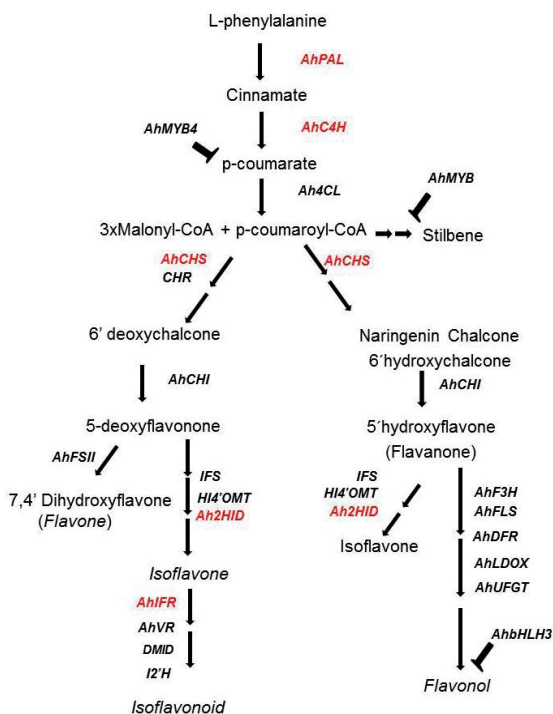
As noted, phenylpropanoids have notable structural and biological function diversity. In terms of defense in plants, phenylpropanoids can be classified into three broad categories according to their function. Those having signaling activity, those known as phytoanticipins, which are part of the basal defensive arsenal of the plant and constitutively accumulate in certain plants tissues, and those whose *de novo* accumulation in plants, as phytoalexins, is induced in response to a biotic aggressor [67,68]. Phenylpropanoids also contribute to human diet and health. Their recognized bioactive properties have acquired medicinal and nutritional importance, mostly due to their antioxidant, antibacterial, antiviral and other reported activities or as effective therapeutic agents against certain types of cancer, cardiovascular pathologies, diabetes, osteoporosis, and neurodegenerative illnesses associated with oxidative stress [43,69,70].

### 3.1. Phenylpropanoid biosynthesis: A profusely branched pathway

Phenylpropanoids contain at least one aromatic ring with one or more hydroxyl groups, and are synthesized via the shikimate pathway alone or in combination with the mevalonate pathway. The first three steps in the synthesis of phenylpropanoid-derived compounds are catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate coenzyme A ligase (4CL), collectively referred to as the general phenylpropanoid pathway (GPP). GPP products then serve as precursors for phenylpropanoid-derived compounds [71,72].

The deamination of phenylalanine to cinnamic acid catalyzed by phenylalanine ammonia lyase (PAL, EC 4-3.1-5) is the initial step shared by all phenylpropanoid secondary metabolites. PAL is a conserved homotetrameric protein that is a key enzyme in the phenylpropanoid pathway of higher plants [73–76]. PAL enzymes are grouped as families having many isoforms that are responsive to different developmental and environmental stimuli [77,78].

Cinnamate is the basic structure from which simple phenylpropanoids with the basic C6–C3 carbon skeleton of phenylalanine are produced, via a series of hydroxylation, methylation, and dehydration reactions. This group includes compounds such as p-coumaric, caffeic, ferulic, and sinapic acids and simple coumarins, which rarely accumulate as free acids inside plant cells, being usually conjugated to sugars, cell wall carbohydrates, or organic acids. Salicylic, benzoic, and other acids are uncharacteristic phenylpropanoids that lack the three-carbon side chain, even though they originate from cinnamate and p-coumarate, whereas a large number of stress-induced phenylpropanoids are derived from the C15 flavonoid skeleton, which is synthesized via the chalcone synthase (CHS)-catalyzed condensation of p-coumaroyl-coenzyme A (COA) and three molecules of malonyl-COA. The tetrahydrochalcone product resulting from the CHS-catalyzed step in most plants is further converted to other flavonoids, such as flavones, flavanones, flavanols, anthocyanins, and 3-deoxyanthocyanidins (Figure 1) [67,68]. Lignin and suberin represent an increased level of complexity, since they are large polymers, constructed from monolignol phenylpropanoid precursors, whose composition varies from species to species.



**Figure 1.** Schematic representation of phenylpropanoid biosynthesis. The image is modified from the original version in [68]. C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; 4CL, 4-coumarate:CoA ligase; DMID, 7,2'-dihydroxy-4'-methoxy-isoflavonol dehydratase; FSII, flavone synthase II; 2HID, 2-hydroxyisoflavanone dehydratase; HI4'OMT, 2-hydroxyisoflavanone 4'-O-methyltransferase; IFR, isoflavone reductase; IFS, isoflavone synthase; I2'H, isoflavone 2'-hydroxylase; PAL, L-phenylalanine ammonia-lyase; VR, vestitone reductase; F3H, flavanone 3-hydroxylase; UFGT, UDP-glucose flavonol 3-O-glucosyl transferase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase. The genes in red text represent those found to be induced by stress conditions in the grain amaranth transcriptomic analysis [14].

### 3.2. Regulation of phenylpropanoid biosynthesis: A complex scenario

The biosynthesis and accumulation of secondary metabolites, including phenylpropanoids, are usually tissue- and developmental-stage-specific. As mentioned above, phenylpropanoids can be present as pigments in leaves, flowers, fruits, and seeds or participate in the establishment of mutualistic or detrimental interactions either with beneficial fungi or bacteria or with pathogenic oomycetes [79]. They can participate in the synthesis of lignins and related fibrous materials associated with changes in the cell wall occurring concomitantly with development, in response to stress [66], or in the determination of pollen function. The latter involves the conjugation of polyamines with hydroxycinnamic acid [63–65,80–82]. Numerous factors mediate the expression of phenylpropanoid genes, including sugar levels, transcription factor



(TF) regulation, and diverse types of stress. Sucrose, for instance, has a dual function, first by providing carbon for phenylpropanoid metabolism, and second, by modulating transcriptional and post-translational regulation of many pigment-related genes [83,84]. Recently, a sugar-related regulatory loop was described in which the induction, by sucrose, of AN1, a MYB TF that activates the phenylpropanoid biosynthetic pathway, was self-regulated by the increased sucrolytic activity induced in parallel by the action of AN1 [85]. Structural variability in secondary metabolism is also determined by post-translational chemical modifications of the primary chemical structure by diverse reactions. This is a mechanism that profoundly alters the biological activity of phenylpropanoid compounds via its ability to modify various critical biochemical parameters, including stability, solubility, and/or localization within the cell. For instance, the glycosylation of hydroxycinnamic acids was found to have an important participation during N-limiting stress conditions in *Populus*, which is a plant known for the great diversity of its phenylpropanoid compounds [86]. Similarly, the glycosylation of diverse secondary metabolites has been shown to be needed to regulate oxidative stress responses in various plant species [87,88]. Moreover, methylation and acylation, for example, can favor the volatilization of secondary metabolites by the generation of esters or ethers. A well-known example is salicylic acid (SA), a phenolic acid which is temporarily transformed to its methyl ester to facilitate its transport to distal tissues in order to regulate systemic signaling during the systemic acquired resistance response to biotrophic pathogens [89,90]. Volatile phenylpropanoid esters can also be found as components of the aroma of fruits such as banana and strawberries [91].

Controlled transcription of biosynthetic genes is one major mechanism regulating secondary metabolite production in plant cells. Several TFs involved in the regulation of metabolic pathway genes have been isolated and studied. Synthesis of more than one class of phenylpropanoid-derived compounds is predominantly under the control of V-myb myeloblastosis viral oncogene homolog (MYB) proteins of the R2R3-MYB class that can act both as transcriptional activators and repressors [92]. The participation of these TFs in many phenylpropanoid-related processes has been extensively recorded in various plant species. In Arabidopsis, several R2R3-MYB members have been implicated as positive regulators of lignin synthesis. For instance, the secondary cell wall-associated AtSND1 protein, in association with related proteins, starts a cascade of events that regulate secondary cell wall formation by inducing the expression of the *AtMYB83* and *AtMYB46* R2R3-MYB genes that then trigger the expression of *AtMYB58*, *AtMYB63*, and *AtMYB85*. These, in turn, upregulate various lignin synthesis genes by interacting with their promoter AC elements [93-96]. Conversely, other R2R3-MYB TFs have been shown to act as repressors of the monolignol pathway in both mono and dicot plant species, thereby leading to the suppression of lignin biosynthesis [97-102]. Anthocyanin pigment synthesis is regulated predominantly by a transcriptional complex consisting of three proteins: (i) R2R3-MYB, (ii) basic-helix-loop-helix (bHLH), and (iii) WD-repeat (WDR), which is known as the MBW complex. The first report describing the formation of a MYB bHLH complex for the activation of anthocyanin biosynthesis was made in maize [103]. Subsequent studies recognized the multiple dependence between MYB TFs (i.e., transparent testa2, TT2), WD40, and bHLH (i.e., TT8) for the regulation of the *BANYULS* gene coding for an anthocyanidin reductase required for proanthocyanidin biosynthesis [104].



Complex formation, initiated by the activation of R2R3-MYB genes can be induced by environmental stress conditions or in response to developmental cues. Known targets of this complex are genes encoding dihydroflavonol 4-reductase (DFR), bHLH2, and, curiously, two MYB repressors whose activation leads to a self-regulatory feedback repression loop [105]. The AtDOF4-2, BrMYB4, and AtMYB4 TFs have been reported also as negative regulators of flavonoid and lignan biosynthesis [80,106,107]. In Arabidopsis, anthocyanin biosynthesis via the MBW complex has been demonstrated to be stimulated in response to light, sucrose, nitrogen, and JA [83,108–110].

In addition, anthocyanin patterning and spatial localization are mainly determined by R2R3-three subgroups of MYB activators, many of which have been identified in plants [92]. The MYB, bHLH, and WDR transcription factors have also been shown to be prevalent in the regulation of proanthocyanidin genes [105,111]. Conversely, the regulation of the flavonol pathway is species-specific, and diverges from the above regulatory mechanisms by its diversity, which may require the action of either a single MYB TF, the formation of an MYB-bHLH dimer or an MBW complex. Additionally, augmented flavonol content in Arabidopsis has been found to result from the association of members of the plant-specific teosinte branched1, cycloidea, and proliferating cell nuclear antigen factor, or TCP TF family that interact with AtMYB12 and AtMYB111 [112]. Additionally, the expression of AtMYB12 in response to both visible and UV-B light is regulated by the basic leucine zipper transcription factor HY5 [113]. Experimental evidence gathered to date clearly indicates that MYB regulation pattern is dependent on cell and tissue type, developmental stage, and environmental conditions. However, information regarding the mechanistic basis of MYB protein responses to biotic and abiotic stimuli remains limited.

### **3.3. Phenylpropanoid and other less abundant secondary metabolites in amaranth: Nutraceutical properties and suggested defensive roles**

Several chemical analyses of diverse tissues of *Amaranthus* species indicate that they differentially accumulate diverse types of secondary metabolites (Table 1). A particular interest in the study of phenolic acids, flavonoids, and other polyphenolics in diverse amaranth species consumed as vegetables and grain has been generated by their high antioxidant activity. As mentioned above, this is mostly because this property has been associated epidemiologically with a decreased risk of diseases associated with oxidative stress, such as cancer and cardiovascular disorder [114]. Consequently, several phenolic acids, flavonoids, and their glycosides have been identified in various *Amaranthus* species (Table 2). These compounds have been isolated using various solvent combinations in various fresh plant tissues obtained from plants at different developmental stages and/ or grown under diverse ambient conditions. They have also been isolated from tissues subjected to diverse processing, from milling to cooking. The concentration of many of these compounds has been observed to vary widely between species and varieties within a species, tissue type, processing and/ or growing conditions, including exposure to (a)biotic stress [3]. A number of selected examples will be described next to illustrate this point.

Metabolic group	<sup>1</sup> cru	hyp	hyb	cau	pan	spi	ret	liv	gan	tri
Flavonoids	<sup>2</sup> +	+	+	+		+				
Betacyanins	+	+	+							+
Phenolic acids	+	+	+	+		+				
Tannins	+	+	+	+		+				
Steroids and triterpenoids	+	+	+	+		+				
Saponins	+	+	+	+		+				
Coumarins					+					
Alkaloids		+				+				
Amarantholidosides				+			+			
Polyhydroxylated nerolidols							+			
Carotenoids	+			+				+	+	
Chlorophylls	+									+
Phytate	+	+		+						
Resinols		+								
Tocopherols and tocotrienols								+		

<sup>1</sup>Amaranthus species included are: **cru**= *cruentus*; **hyp**= *hypochondriacus*; **hyb**= *hybridus*; **cau**= *caudatus*; **pan**= *paniculatus*; **spi**= *spinosus*; **ret**= *retroflexus*; **liv**= *lividus*; **gan**= *gangeticus*, and **tri**= *tricolor*.

<sup>2</sup>The + sign represents species in which these compounds have been detected.

**Table 1.** The diversity of secondary metabolites in amaranth.

Compound	<sup>1</sup> hyp	cru	cau	hyb	man	spi	pan	ret	tri	gan
Total phenolic acids	<sup>2</sup> S, F	S, V	S							
Gallic acid	F, L	S, Sp		L			S			
Vanilic acid	F, S, L	S		L	S					
Cinnamic acid		S								
Syringic acid	Fl, S	Sp								
p-Coumaric acid	S, L	S, Sp, L	S	L	S		S			
Ferulic acid	Fl, L	L, S, Sp	S, IF	L			S			
Protocatechuic acid	S	S	S, Sp		S		S			
p-Hydroxybenzoic acid	Fl, S, L	S	S	L	S		S			
Caffeic acid	L	L, S	S	L			S			
Chlorogenic acid	L			L						

Compound	<sup>1</sup> hyp	cru	cau	hyb	man	spi	pan	ret	tri	gan
<i>Sinapic acid</i>	L			L			S			
<i>Salicylic acid</i>	L		S	L			S			
<i>Caffeoylquinic acids</i>							St			
<i>Cumaroylquinic acids:</i>							St			
<i>Feruloylquinic acids</i>							St			
<i>Hydroxycinnamates (quinic acid)</i>	L			L		St, L				
<i>Flavonoids</i>	Fl, S	L		L						
<i>Rutin</i>	S, L, St, Fl	S, L, St, Fl, Sp	S, L, St, Fl	S, L, St, Fl		WP, St		S, L, St, Fl	S, L, St, Fl	
<i>Isoquercetin</i>	S, L								L	
<i>Quercetin: released from rutin</i>	S, L, St, Fl	S, L, St, Fl	S, L, St, Fl	S, L, St, Fl		L		S, L, St, Fl		L
<i>Quercetin diglycoside</i>						St				
<i>Quercetin-3-O-glucoside</i>						St				St
<i>Nicotiflorin</i>	S	S			S					
<i>Vitexin</i>		S								
<i>Isovitexin</i>		S				St				
<i>Orientin</i>		Sp								
<i>Morin</i>		Sp								
<i>neohesperidin and hesperidin</i>		Sp								
<i>Kaempferol diglycoside</i>					L	St				
<i>Tannins</i>	S, L	S, L								
<i>Polyphenolics</i>	S, L	S, L		S, L						
<i>Anthocyanins</i>	S									
<i>Betains, betacyanins</i>	L	L			L				L	
<i>Phenolic Amides</i>	L				L					L

<sup>1</sup>Amaranthus species included are: **hyp** = hypochondriacus; **cru** = cruentus; **cau** = caudatus; **hyb** = hybridus; **man** = mantegazzianus; **spi** = spinosus; **pan** = paniculatus; **ret** = retroflexus; **tri** = tricolor, and **gan** = gangeticus.

<sup>2</sup>Tissues or plant developmental stage examined include: **F** = flower; **Fl** = seed flour; **L** = leaf; **S** = seed; **Sp** = sprout; **St** = stem, and **WP** = whole plant.

**Table 2.** Diversity of phenylpropanoid metabolites reported in different *Amaranthus* species (modified and amended from [3])

A recent study reported a significantly variable content of bioactive substances and phenolic contents in leaves of various cultivars of *A. tricolor* and *A. hypochondriacus* [49]. For instance, leaf color attributes and betacyanins varied widely among the cultivars. Also, the hyperoside flavonoid was found only in one *A. hypochondriacus* cultivar, in contrast to isoquercetin and rutin, which were abundantly found in all amaranth cultivars examined. SA, syringic, gallic, vanilic, ferulic, p-coumaric, and sinapic acids were also common phenolic acids detected in all amaranth cultivars, whereas significant amounts of ellagic and sinapic acids were only detected in *A. hypochondriacus* cultivars. Lastly, total phenol content was found to be strikingly greater than total phenol index in *A. tricolor*. The free phenolic acid profile in seed ethanol extracts isolated from *A. caudatus* and *A. paniculatus* was also found to be significantly different [115]. However, the differences observed had only a slight influence on their antioxidant activity. A subsequent study performed with seeds of *A. cruentus* showed that processing (i.e., popping or flaking) and cultivation area had significant effects on their total phenolic acid content, whereas differences in individual phenolic acids (e.g., ferulic acid in processed seeds) were highly variable and were found not to have statistical significance [116]. A similar influence of climatic and agro-technical factors on the polyphenol content of amaranth seeds was described prior to this report [117].

In this study, the levels of 11 different polyphenols, including three flavonoids, i.e. rutin, isoquercitrin, and nicotiflorin, and 8 phenolic acids, i.e., protocatechuic, vanillic, 4-hydroxybenzoic, p-coumaric, syringic, caffeic, ferulic, and salicylic acids, were analyzed in mature seeds of 18 *Amaranthus* genotypes, including *A. cruentus*, *A. hypochondriacus*, *A. mantegazzianus*, and one grain amaranth hybrid. All were cultivated in three different countries in two continents and in two different locations within the same country. Interestingly, the results derived from principal component analysis (PCA) showed that varying environmental conditions had a contrasting effect on the rutin and nicotiflorin flavonoid levels in the seeds, which largely affected the former. In contrast to the above study, individual phenolic acids in seed samples, such as p-coumaric and protocatechuic acids, were found to be descriptors of climatic and other variations between the different locations studied. Besides, genotype-dependent effects were also observed, since polyphenols content in *A. hypochondriacus* displayed the lowest variation between cultivation sites and the highest content of flavonoids. Comparable results were obtained from the analysis of the aerial tissues of *A. mantegazzianus* and the grain amaranth hybrid plants grown in the same locations. However, this study included the determination of additional compounds such as hydroxycinnamyl amides (N-trans-feruloyltyramine and N-transferuloyl- 4-O-methyldopamine) and betaines (glycinebetaine and trigonelline) [118].

Once more, PCA clearly identified that samples from one location (i.e., in Argentina) differed from all other experimental sites by having a higher content of most compounds analyzed. Phenolic acids were, once again, a key group of compounds since their analysis permitted the separation of the different experimental groups, while separation of both amaranth genotypes could be performed primarily by the higher contents of trigonelline and the two hydroxycinnamyl amides present in *A. mantegazzianus*. Additionally, the contents of polyphenols and betaines in the aerial parts of grain amaranth were found to be very dependent on growing

conditions. Further analysis revealed that trigonelline and the two hydroxycinnamyl amides could be tentatively used for chemo-taxonomic classification. Mention must be made of the presence of these and other cinnamoylphenethylamines (i.e., caffeoyltyramine, feruloyldopamine, sinapoyltyramine, p-coumaroyltyramine, and feruloyl-4-O-methyldopamine), which had been just previously reported in the Amaranthaceae for the first time [119]. Feruloyldopamine was different from the other cinnamoylphenethylamines detected by its seemingly ubiquitous presence in the genus *Amaranthus* and to its moderate antifungal activity. In addition to these effects, cinnamoylphenethylamines were previously associated with various other biological activities, such as the potentiation of antibiotics and inhibition of prostaglandin biosynthesis. In the same context, a recent study also determined that leaves and flowers of *A. hybridus*, as well as their extracts, possessed higher antioxidant activities compared to stems and seeds. An on-line HPLC- 2, 2-diphenyl-1-picrylhydrazyl radical assay determined that rutin was the main radical scavenger in these amaranth tissues/extracts, compared to other phenolic compounds detected, such as nicotiflorin, isoquercitrin, 4-hydroxybenzoic, and p-coumaric acids [38].

The influence of other experimental effects on polyphenol levels, such as tissue type, ripeness, or time of harvest was demonstrated by results obtained from a two-year field study performed with various grain and foliar amaranth species, in addition to two amaranth hybrids [120]. The tissue-type effect was clearly demonstrated by results that showed a more than 300-fold difference in rutin content between seeds and leaves. This study also showed that rutin was predominantly found in mature amaranth leaves, in accordance with previous reports describing a progressive accumulation of rutin in maturing amaranth and other rutin-accumulating plants, such as common buckwheat. Genotype-dependent effects were again observed, since noticeable variations between the species and even between the varieties belonging to the same species were detected. For instance, the highest rutin contents found in *A. retroflexus* leaves examined just before harvest contrasted with those measured in *A. tricolor*, which had approximately 12-fold and 67-fold lower rutin contents in leaves and flowers of plants sampled at the same developmental stage, respectively.

Other environmental factors, such as light, or the lack of it, have been also found to selectively influence the level of certain phenolic compounds. Thus, growth of *A. cruentus* sprouts in plain daylight had no effect on gallic acid content but increased the amount of rutin, whereas growth in darkness led to the accumulation of vitexin and isovitexin [121]. In related studies, the antioxidant activity and related color parameters were analyzed in *A. tricolor* and other pigmented leafy vegetables when grown under different photoperiods and light intensities. Betacyanin, chlorophyll, total polyphenol, and antioxidant activity peaked in leaves of *A. tricolor* plants maintained under a 12 h photoperiod but were severely reduced when exposed to constant light for 24 h. The quality and intensity of the light were also found to be significant factors, since shading by blue polyethylene sheets increased betacyanins, polyphenols, and antioxidant activity [122–124]. Sample preparation and the subsequent extraction and measurement protocols are factors that are also known to significantly affect the analyses of polyphenols content in amaranth species. Several studies, for example, have reported a wide range of tannin content in seeds and leaves of various grain and vegetable amaranth species [125,126].

Aside from expected genotype- and tissue-related variations, the differences observed were also suggested to be caused by the analytical methods employed for tannin content determination. Posterior processing procedures (e.g., roasting of seeds, cooking, or blanching of leaves, etc.) were also found to be significant factors affecting tannin content in amaranth seeds or leaves. Subsequently, a pertinent study assessed that the nutraceutical value of leaves of *A. cruentus* and *A. hybridus*, measured in terms of *in vitro* antioxidant (provided mostly by the presence of polyphenols, tannins, flavonoids, and betalains) and xanthine oxidase inhibitory activities, was highly dependent on the type of solvents used for extraction [47]. Similar variations were obtained in another study in which the combined effect of the extraction method and type of solvent on the antioxidant capacity and total phenolic content of extracts from seeds or leaves of *A. hypochondriacus* was evaluated [127]. In this respect, the extraction of phenolic compounds from seeds is believed to be affected by the complex interaction existing between phenolic acids and their cell wall constituents. For example, an investigation of the association of ferulic acid, an alkali-extractable phenolic acid, with the dietary insoluble fiber and non-starch polysaccharides of seeds of *A. caudatus* led to the identification of three complex compounds: O-(6-O-trans-feruloyl- $\beta$ -D-galactopyranosyl)-(1  $\rightarrow$  4)-D-galactopyranose, O-(2-O-transferuloyl- $\alpha$ -L-arabinofuranosyl)-(1  $\rightarrow$  5)-L-arabinofuranose, and O- $\alpha$ -L-arabinofuranosyl-(1  $\rightarrow$  3)-O-(2-O-trans-feruloyl- $\alpha$ -L-arabinofuranosyl)-(1  $\rightarrow$  5)-L-arabinofuranose. This study also demonstrated that ferulic acid in amaranth seed cell walls is predominantly bound to pectic arabinans and galactans [128]. Additionally, the presence of ferulate associations with polysaccharides of dietary fibers could be considered to have taxonomic potential based on a previous study in which ferulate cross-links in the cell walls of dietary fibers were found to be restricted to species belonging to families of the Caryophyllales [129].

Two recent reports focused on direct or indirect changes in polyphenolic content in grain amaranth plants exposed to different stress conditions. The first one determined changes in the abundance of 3 flavonoid glucosides (rutin, nicotiflorin, and isoquercitin), 9 phenolic compounds (coumaric, vanillic, caffeic, syringic, ferulic, sinapic, protocatechuic, salicylic, and 4-hydroxybenzoic acid) and 3 betalains (amaranthine, iso-amaranthine, and betanin) in leaves of five varieties of three grain amaranth species subjected to insect folivory, in a one-year field trial [44]. Multivariate regression analysis revealed significant and predictable differences in the chemical composition of the leaves between grain amaranth genotypes. A similar analytical approach indicated that 8 of the 15 compounds analyzed in the plants, including all 3 flavonoid glucosides, 2 betalains, and 3 phenolic acids, had significant linear relationships with insect herbivory in the field. However, the experiment was not designed to determine biological relevance of the herbivory-induced accumulation of some of these metabolites in amaranth leaves. Thus, the possibility that phenolics could have been acting as feeding deterrents, phagostimulants, digestion inhibitors, digestion stimulants, toxins, toxicity reducers, signal inhibitors, and/or signal transducers in damaged grain amaranth remained unanswered [130]. In potato (*Solanum tuberosum*), for instance, rutin is known to accumulate to high levels only in varieties which are resistant to *Pectobacterium atrosepticum*, a very destructive necrotrophic bacterial pathogen. On the other hand, rutin is considered to be a susceptibility factor with respect to *Phytophthora infestans* infections [131].

In a related proteomic study, the upregulation of transcription factors (i.e., DOF and MIF) was found to be coupled with the downregulation of caffeic acid O-methyltransferase, an isoflavone reductase-like protein, and two different S-adenosylmethionine synthetases, which are enzymes related to secondary metabolism associated with flavonoid and lignin synthesis [23]. Based on these results, the authors suggested that repressed root growth in grain amaranth plants subjected to severe drought is an adaptive response occurring in response to decreased root lignification. This proposal is in accordance to other reports showing that roots of plants exposed to different stresses may change their lignin content and composition [132]. One possible advantage derived from reduced lignification of the roots, particularly in the elongation zone, is that it may facilitate growth recovery once drought stress has been alleviated [133].

### 3.4. Secondary metabolism biosynthetic pathways and related genes in amaranth: Wandering into unknown territory?

The information provided above indicates that a potentially high nutritive and medicinal benefit may be derived from the consumption of amaranth seeds and foliage, which are high in antioxidant phenolic compounds, among other health-enhancing constituents. Until recently, information about the biosynthesis of these bioactive compounds and of the genes coding for the respective biosynthetic enzymes was practically null in amaranth. However, a recent transcriptomic study of grain amaranth leaves subjected to various stress treatments [14] revealed the presence of several genes involved in secondary metabolism, mostly in the phenylpropanoid biosynthetic pathway. The rest of this chapter will concentrate on the description of their characteristics and possible functions.

The transcriptomic study permitted the identification of 95 genes that code for the enzymes that most probably form part of the secondary metabolite biosynthetic flow in *A. hypochondriacus*. These are shown in Table 3. The transcriptomic data also uncovered the presence of several TFs that could be involved in the regulation of distinct branches of the secondary metabolism biosynthetic processes in grain amaranth, including the phenylpropanoid pathway.

Metabolic Group	Putative gene	<sup>4</sup> No	Function
ID <sup>1</sup>	Indole 3-glycerol phosphate lyase ( <i>AhIGL</i> )	1	Emission of volatile compounds related to defense [169].
ID <sup>1</sup>	Anthranilate synthase ( <i>AhANS</i> )	2	Production of tryptophan pathway metabolites as part of an Arabidopsis defense response (e.g., camalexin) [170, 171].
ID <sup>1</sup>	<i>AhWRKY33</i> , transcription factor	1	Pathogen-induced transcription factor; camalexin biosynthesis [172].
A <sup>2</sup>	Berberine bridge enzymes ( <i>AhBBE</i> )	3	Upregulation of these genes was observed at specific stages of development and in response to osmotic stress and pathogens attack [173,174].
A <sup>2</sup>	Strictosidine synthase like ( <i>AhSSL</i> )		The four SSL-coding genes in Arabidopsis are regulated individually, suggesting specific roles in

Metabolic Group	Putative gene	<sup>4</sup> No	Function
			basal (SSL4) and inducible (SSL5-7) plant defense mechanisms [175].
A <sup>2</sup>	Tropinone reductase ( <i>AhTR</i> )	4	The <i>TR-I</i> gene expression was found to be upregulated on exposure to signal molecules (MeJA and SA) and in response to mechanical injury [176].
A <sup>2</sup>	Hyoscyamine 6-hydroxylase ( <i>AhH6H</i> )	2	The overexpression of <i>H6H</i> increases scopolamine production [177].
T <sup>3</sup>	Acetoacetyl-CoA thiolase ( <i>AhAACT</i> )	2	The AACT is a regulatory enzyme in isoprenoid biosynthesis involved in abiotic stress adaptation [178].
T <sup>3</sup>	3-Hydroxy-3-methyl-glutaryl-CoA reductase ( <i>AhHMGR</i> )	3	HMGR activity induced by wounding, elicitor, or pathogen challenge is correlated with increased <i>HMGR</i> mRNA. The mutant <i>hmgr1</i> shows early senescence and sterility, as well as a dwarf phenotype [179, 180].
T <sup>3</sup>	1-Deoxy-xylulose 5-phosphate synthase ( <i>AhDXS</i> )	1	<i>DXS</i> genes type II been proposed to be primarily involved in defense responses and secondary metabolism [181, 182].
T <sup>3</sup>	Sesquiterpene synthase ( <i>AhSS</i> )	2	Involved in indirect defense of maize against herbivore attack [183].
T <sup>3</sup>	Squalene synthase ( <i>AhSqS</i> )	1	The downregulation of <i>SqS</i> reduced stomatal conductance and conserved more moisture; it also led to increased grain yield and improved drought tolerance [184].
T <sup>3</sup>	Squalene epoxidase ( <i>AhSqE</i> ) y β-amyrin synthase ( <i>AhβAS</i> )	4	The synthesis of saponins and expression of respective genes ( <i>squalene synthase</i> , <i>squalene epoxidase</i> , and <i>β-amyrin synthase</i> ) is induced in response to elicitors or MeJA treatment [185,186].
T <sup>3</sup>	<i>AhWRKY1</i> , transcription factor	1	The orthologous genes, <i>GaWRK1</i> , regulates the expression of δ-cadinene synthase needed for the synthesis for phytoalexin gossypol. This gene is also part of the defensive responses to pathogens [187,188].
<sup>1</sup> ID: Indole derivatives			
<sup>2</sup> A: alkaloids			
<sup>3</sup> T: terpenes			
<sup>4</sup> Number of genes detected in the grain amaranth transcriptome [14]			

**Table 3.** Secondary metabolism genes identified in grain amaranth



In this respect, the phenylpropanoid pathway is the best represented, with more than 69.5% of the above 95 biosynthesis-related genes coding for enzymes related to their biosynthesis [14]. Their position in the intricate phenylpropanoid biosynthetic reaction pathway is shown in Figure 1. Many of these genes have been amply characterized in other plant species. However, those described below code for enzymes that should be highly active, considering that, as mentioned in previous sections of this chapter, amaranth plants have been frequently found to be unusually rich in these compounds (Table 4). Thus, these particular grain amaranth enzymes and/or genes could have attractive biochemical and/or regulatory properties that could offer potentially important biotechnological applications. In addition, four TFs similar to those described above as key regulators of this biosynthetic pathway are described.

Flavonoids (µg/mg)	<i>Zea mays</i>	<i>Sorghum sp.</i>	<i>Amaranthus sp.</i>
Catequin	0.15-0.23	0.07-0.22	-
Rutin	0.38-0.47	0.17-0.30	4.0-10 (4798-15531)*
Quercetin/isoquercetin	-	0.1-0.65	0.3-0.5 (27.8-279.5)*
Nicotiflorin	-	-	4.8-7.2 (141-1281)*

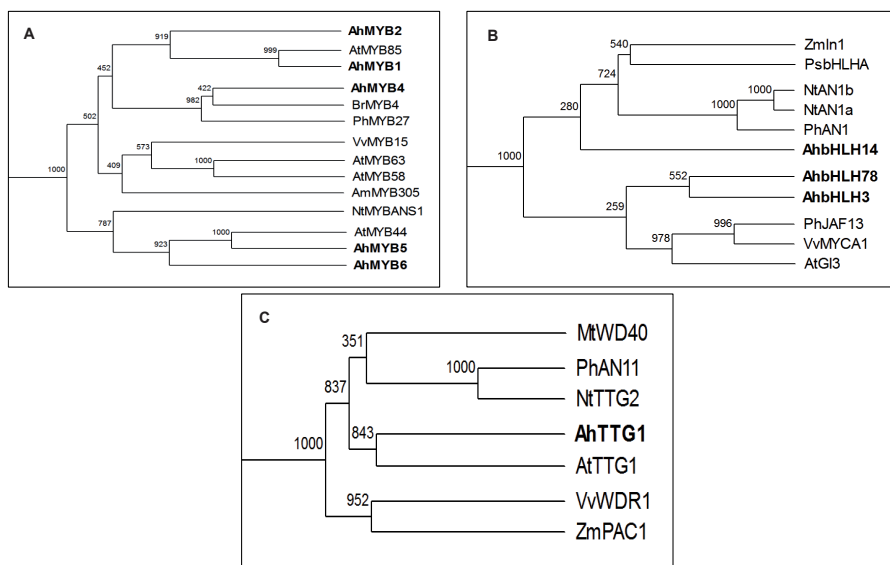
\*Numbers in parentheses indicate the quantity of these compounds detected in leaves

**Table 4.** Flavonoid content in amaranth, compared to other grain-producing C4 species

As already mentioned, PAL catalyzes the first committed step of the phenylpropanoid pathway, which is shared by all compounds produced by downstream ramifications of the pathway (Figure 1). It also represents a bifurcation point between primary and secondary metabolism. Diverse environmental stimuli and developmental programs regulate PAL. It is induced by lignin demands for cell wall fortification and by both biotic (e.g., pathogen and insect damage) and abiotic stresses (e.g., UV irradiation, low temperatures, and nutrient deficiency) [134,135]. PAL activity has been found in all the higher plants analyzed so far, and in some fungi and a few bacteria, but not in animals. In all species studied, *PAL* is part of a multigenic family. For instance, four *PAL* genes have been described in *Arabidopsis*, five in *Populus trichocarpa* and tomato (*Solanum lycopersicum*) and sixteen in potato (*S. tuberosum*). Two genes have been identified so far in *A. hypochondriacus* [14]. In *Arabidopsis*, the induction of *PAL* genes is closely related to a defense-related accumulation of flavonoids [136]. Similarly, the induced expression of *AhPAL1* in *A. hypochondriacus* and *A. cruentus* plantlets pre-treated with the BTH, a priming agent, and subsequently infected by *Clavibacter michiganensis* or various *Pseudomonas syringae* pathovars, all of which are opportunistic but highly damaging aggressors of grain amaranths, could contribute the increased resistance observed [15]. However, the exact relationship of this gene with defense-related accumulation of phenylpropanoid compounds and/ or cell wall fortification via lignin incrustations has not been evaluated.

A number of R2R3 MYB transcription factors are known to be able to transactivate *PAL* promoters to control the tissue-specific expression of this gene. An *in silico* analysis of the 64

MYB TF identified in grain amaranth revealed, however, that only five of them are candidates to participate in the phenylpropanoid pathway. In common with orthologs identified in other plants species (Figure 2), these amaranth TFs may also activate the phenylpropanoid pathway via the activation of *PAL* expression [135]. Available transcriptomic data support their role in the regulation of stress responses. Thus, AhMYB5 (Figure 2A) was found to be induced by bacterial infection, which coincided with the participation of AtMYB44, its homolog in *Arabidopsis*, in the regulation of resistance responses against pathogens and/or water and salt stress [14,137,138]. However, nothing is known in amaranth regarding the interaction of these MYB TFs with other regulatory components, such as certain bHLH and WD40 proteins. Further *in silico* analysis of amaranth transcriptomic data revealed the presence of 50 genes belonging to either of the two gene families. However, only AhbHLH3, whose nomenclature is indicative of its similitude with a similarly named ortholog TF present in *Beta vulgaris* (Figure 2B), was found to have the potential to participate in the regulation of the phenylpropanoid pathway in addition to a possible role in the control of the JA-dependent wound response, via its potential interaction with JAZ proteins [139].



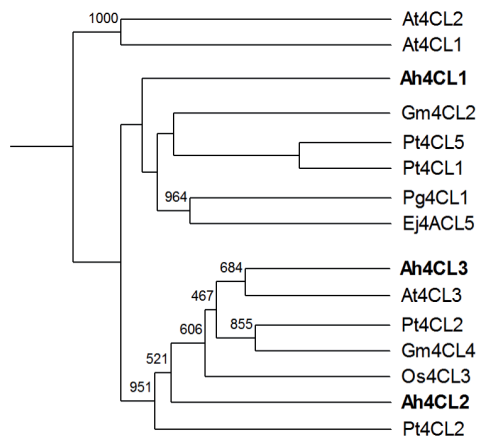
**Figure 2.** Phylogenetic trees of transcription factors from amaranth and other species: MYB (A), bHLH (B), and WD40 (C). *Amaranthus hypochondriacus*: AhMYb1, AhMYb2, AhMYb4, AhMYb5, and AhMYb6; AhbHLH3, AhbHLH14, and AhbHLH78, and AhTTG1. *Arabidopsis thaliana*: AtMYB85 = AEE84639, AtMYB63 = NP\_178039.1.1, AtMYB58 = EFH69169.1, AtMYB44 = NP\_201531, AtGL3 = NP\_680372, AtTTG1 = AED93321.1; *Brassica rapa*: BrMYA4 = ADZ98868.1; *Petunia hybrida*: PhMYB27 = AHX24372.1, PhAN11: AAC18914, PhAn1: AAG25927, PhJAF13: AAC39455; *Vitis vinifera*: VvMYB15 = AHA83524.1, VvMYCA1 = ABM29332, VvWDR1 = ABF66625; *Zea mays*: ZmIn1 = AAB03841, ZmPAC1: AAM76742; *Pisum sativum*: PshHLHA = ADO13282; *Nicotiana tabacum*: NtAN1a = AEE99257; NtAN1b = AEE99258, NtTTG2 = ACN87316; *Medicago truncatula*: MtWD40: ABW08112

Correspondingly, the *AhTTG1* gene, which codifies for a protein having a conserved WD40 domain, shared 77% homology with the Arabidopsis *AtTTG1* gene (Figure 2C). Based on the observed similarity, it could be hypothesized that *AhTTG1* could participate in flavonol (quercetin)-dependent developmental processes, such as the control of root growth under abiotic stress conditions [140].

Cinnamate 4-hydroxylase (C4H) is the second key enzyme in the phenylpropanoid pathway and catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid, which is the biosynthetic precursor of flavonoids, phytoalexins, lignin, pigments, and many other defense-related compounds. C4H belongs to the large CYP73 family of cytochrome P450 monooxygenases (P450s). In addition to its central role in the phenylpropanoid biosynthetic pathway, it is also involved in the biosynthesis of various other compounds (e.g., fatty acids, alkaloids, and terpenoids) and may participate in the detoxification of herbicides and pesticides. In accordance with many key biosynthetic genes, *C4H* genes form extensive multi-gene families in various plant species, including *Populus*, orange, and pea [141–143]. Several studies have shown that *C4H* genes have a tissue-specific expression pattern, and that similar to *PAL* genes, they may be also induced by wounding, pathogen infection, and nutrient deficiency [144–147]. Four genes, *AhC4H1–4*, have been identified in grain amaranth. Based on transcriptomic and other data, only the *AhC4H2* is induced by (a)biotic stress [14,134]. However, nothing is known regarding its participation in the synthesis of phenylpropanoids, and perhaps other compounds, in grain amaranth.

The enzyme 4-coumaric acid CoA ligase (4CL) plays an important role in the biosynthesis of lignin precursors such as hydroxycinnamate-CoA thioesters. The *4CL* gene is differentially expressed during development in various tissues and presents multiple isoforms with different substrate specificities. In Arabidopsis, four *4CL* isozymes have been identified. Of these, *4CL1* and *4CL2* are known to be involved in the lignin biosynthesis, while *4CL3* participates in flavonoid and other non-lignin biosynthesis pathways. In *P. trichocarpa*, 17 genes showing sequence similarity with known *4CLs* were identified, whereas a similar *4CL1* enzyme was detected in developing xylem tissues of *P. tremuloides*. In addition, *Ptr4CL2* was proposed to be involved in flavonoids biosynthesis. Their role in lignin biosynthesis was demonstrated by reports showing that the downregulation of *4CL1* in Arabidopsis, *4CL1* in poplar, and *4CL3* in rice resulted in reduced lignin content [134]. The *4CL* gene family is divided into two main groups. Group II contains those genes which are associated with flavonoid biosynthesis, including two that were identified in grain amaranth: *Ah4CL2–3* (Figure 3). Conversely, the *Ah4CL1* gene could be part of group I and, thereby, participate in lignin biosynthesis (Figure 3). Numerous studies have reported the induction of *4CL* genes by wounding, UV radiation, and pathogen infection in potato, soybean, Arabidopsis, and rice [148–153].

Chalcone synthase (CHS) is another key enzyme of the flavonoid/ isoflavonoid biosynthesis pathway. Besides being part of the plant developmental program, *CHS* gene expression is induced in plants exposed to diverse stress conditions such as UV light, excess salinity, insect herbivory, and bacterial or fungal infection. *CHS* expression leads to the accumulation of flavonoid and isoflavonoid phytoalexins and is involved in the SA defense pathway. Multiple

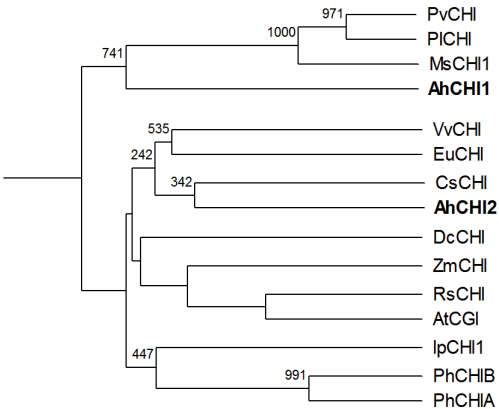


**Figure 3.** Phylogenetic tree of genes encoding 4-coumarate CoA ligase from amaranth and other species. *Amaranthus hypochondriacus*: AhCL1, AhCL2, and AhCL3. *Arabidopsis thaliana*: At4CL1 = U18675, At4CL2 = B1GUZ3, At4CL3 = AY376730; *Glycine max*: Gm4CL2 = X69954, Gm4CL4 = X69955; *Populus tremuloides*: Pt4CL1 = AF041049, Pt4CL2 = AF041050, *Populus trichocarpa*: Pt4CL5 = EU603299; *Populus generosa*: Pg4CL1 = AF008184; *Ej4ACL5* = EF685345; *Oryza sativa*: Os4CL3 = L43362; *Pinus taeda*: Pt4CL2 = U12013.

copies of the CHS gene have been detected in several plants including *Gerbera hybrida*, *Petunia hybrida*, *Ipomoea sp.*, *Cannabis sativa*, and *Pisum sativum*. In contrast to grain amaranth, in which two CHS genes (*AhCHS1-2*) have been identified, only single copies of the CHS gene have been found in *Arabidopsis*, parsley, and snapdragon. The structural and catalytic domains present in the hypothetical AhCHS1-2 enzymes are shown in Figure 4. Chalcone isomerase (EC 5.5.1.6), CHI, is one of the most important intermediate enzymes in the flavonoid pathway whose activity involves the modification of substrates previously synthesized by CHS. Although these modifications can occur spontaneously, the efficiency of the reactions are  $10^7$ -fold higher if catalyzed by CHI. A consequence of CHI activity is the typical lack of chalcones and naringenin chalcone in plants, due to their rapid isomerization to naringenin by this enzyme. However, other reactions such as the isomerization of 6-deoxychalcone to 5-deoxyflavanone are rather slow because of the intramolecular hydrogen bond in the substrate molecule. CHIs are classified into two types, and their distribution is highly family-specific. CHIs generally found in non-legumes exclusively catalyze the isomerization 6-hydroxychalcone to 5-hydroxyflavanone. CHIs with this catalytic function are referred to as type I CHIs. On the other hand, most, if not all, of the CHIs found so far in leguminous plants (referred to as type II CHIs) recognize both 6-deoxychalcone and 6-hydroxychalcone as substrates, yielding 5-deoxyflavanone and 5-hydroxyflavanone, respectively. Although more additional information is needed, available data indicate that each of the two *AhCHI* genes (i.e., *AhCHI1-2*) identified in grain amaranth may belong to the different types described, as shown in Figure 5. This characteristic could allow the synthesis of an increased diversity of phenylpropanoid metabolites in amaranth and in related species [134].

AhCHS1	LKFLDWVKLLLRPSRSGANLSQKITHVIMCTTSGVDMPGADYQLTKLLGLRPSVRRFML
AhCHS2	-----PGADYQLTKLLGLRPSVRRFML
MsCHS	-----ITHLIVCTTS-VDMPGADYQLTKLLGLRPVVKRYMM
AhCHS1	YQGGCFAGGTVLRLAKDLAENNNGARVLVVCSEITAICFRGPTETHLDSMVGQALFGDGA
AhCHS2	YQGGCFAGGTVLRLAKDLAENNNGARVLVVCSEITAICFRGPTETHLDSMVGQALFGDGA
MsCHS	YQGGCFAGGTVLRLAKDLAENNGARVLVVCSEVTAVTFRGPSTHLDLSLVGQALFGDGA
AhCHS1	GALIVGADPDELIERPLFKMVWTAQTILPDSEGAIDGHLREVGLTFHLLKDVPGLISKNI
AhCHS2	GALIVGADPNEPIERPLFKMVWTAQTIIIPDSEGAIDGHLREVGLTFHLLKDVPGLISKNI
MsCHS	AALIVGSDPVEIEKPIFEMVWTAQTIAPDSEGAIDGHLREAGLTFHLLKDVPGLIVSKNI
AhCHS1	NKALEDAFLPLGINDWNSIFWIAHPGGPAILDQVEQKLGLENEKLTATRNVLRDYGNMSS
AhCHS2	NKALEDAFNPFINDWNSIFWIAHPGGPAILDQVEEKLGDKELKATRNVLRDYGNMSS
MsCHS	NKALVEAFEPLGISDYSIFWIAHPGGPAILDQVEQKALKPEKMKATREVLSEYGNMSS
AhCHS1	ACVLFILDEMRKKSSEEGKATTGEGLDWGVLFGFGPGLTVETVVLHSVPLNY-
AhCHS2	ACVLFILDEMRKKSCEGKSTTGEGLHWGVLFGFGPGLTVETVVLQSVPIAET
MsCHS	ACVLFILDEMRKKSQDGLKTTGEGLEWGVLFGFGPGLTIETVVLRSVTI---

**Figure 4.** Alignment of chalcone synthase enzymes identified in grain amaranth (AhCHS1-2) and *Medicago sativa* (MsCHS). The amino acid residues found in the catalytic site are shown in red, whereas those that compose the structural domains are highlighted in yellow [203].



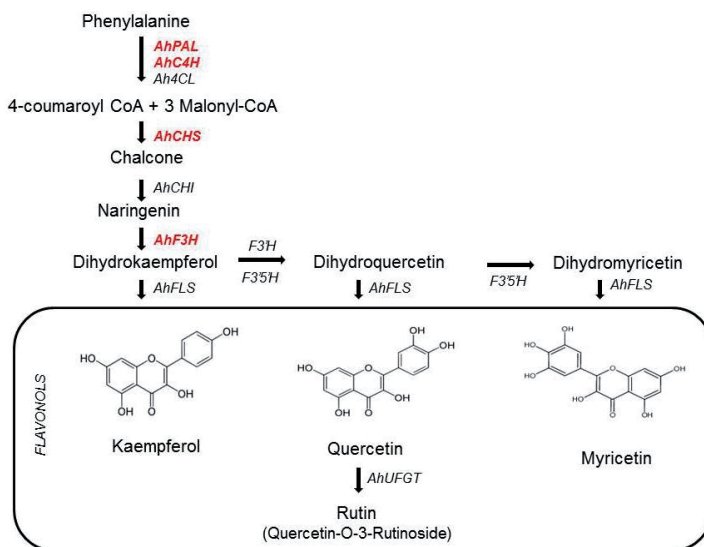
**Figure 5.** Phylogenetic tree of genes encoding chalcone isomerase from amaranth and other species. *Amaranthus hypochondriacus*: AhCHI1 and AhCHI2. *Zea mays*: ZmCHI = Z22760.1; *Medicago sativa*: MsCHI1 = M31079.1; *Pueraria lobata*: PICH1 = D63577.1; *Phaseolus vulgaris*: PvCHI = XM\_00712628.1; *Arabidopsis thaliana*: AtCGI = M86358; *Raphanus sativus*: RsCHI-AF031921.1; *Petunia hybrida*: PhCHIA = AF233637.1, PhCHIB = X14590.1; *Ipomoea purpurea*: IpCHI1 = AF028238.1; *Dianthus caryophyllus*: DcCHI = Z67989.1; *Elaeagnus umbellata*: EuCHI = AF061808.1; *Citrus sinensis* CsCHI = AB011794.1; *Vitis vinifera*: VvCHI = X75963.1.

Flavanone 3-hydroxylase (EC 1.14.11.9), F3H, is a key enzyme in the flavonoid biosynthetic pathway, catalyzing the 3-hydroxylation of (2S)-flavanones, such as naringenin to dihydroflavonols. In soybean seeds the downregulation of *F3H* is accompanied by increased accumulation of isoflavonoids [154]. Seven copies of the *F3H* gene have been identified in wheat, barley, and rye. *F3H* activity has been detected in young flower petals and its expression is associated with disease resistance in plants [155]. The antisense repression of the *F3H* gene in carnation flowers (*Dianthus caryophyllus*) and strawberries reduced anthocyanin levels while compounds such as methyl benzoate and 2-hydroxymethyl benzoate (responsible for the flower's fragrance) or polyphenols, including p-coumaroyl-glucose and p-coumaroyl-1-acetate among many others, were found to accumulate [156,157].

Tolerance to UV radiation and severe water deprivation in the extremophyte *Reaumuria soongorica* was associated with increased expression and enzymatic activity of *F3H*. This increase was also correlated with flavonoid accumulation in consequent antioxidant activity [155]. In grain amaranth, the *AhF3H1* gene, sharing a 77% of homology with the *F3H* gene in *R. soongorica*, was identified. This finding suggests that this gene could be an important factor in the proposed role played by the phenylpropanoid pathway in the tolerance mechanisms used by extremophytes, many of which belong the Caryophyllales order, to thrive in the highly adverse environmental they inhabit [1,158-160]. The use of this gene, and perhaps others related to the phenylpropanoid biosynthetic pathway, offers the attractive possibility of their application for biotechnological purposes in commercial crops.

Flavonol synthase (EC 1.14.11.23), FLS, is another relevant enzyme due to its crucial participation in the conversion of several precursors needed for different branches of the flavonoid biosynthesis. For instance, the biosynthesis of flavonols from dihydroflavonols is catalyzed by FLS, a soluble 2-oxoglutarate-dependent dioxygenase (2-ODD) (Figure 6). The expression is tissue- and organ-specific organ and is regulated by various light intensities, pathogen infection, and herbivore attack [134,161-163]. Silencing of the *FLS* gene in tobacco led to suppression of flavonol content through a decrease in transcript level of flavonol synthase. Moreover, silencing of flavonol synthase diverted the pathway toward catechin and epicatechin production through enhanced expression of genes encoding dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS). Curiously, it also increased the activity of ROS-detoxifying enzymes such as glutathione reductase (GR), APX, and CAT. Grain amaranth transcriptomic data showed that a related gene, *AhFLS2*, was induced by both drought and salinity stress. The above information suggests that this gene is a promising candidate for crop improvement, acting as a controller of stress-related responses, perhaps by its participation in the biosynthesis of catechin and the regulation of other protective biochemical processes that could help ameliorate oxidative stress in plants [164].

Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219), DFR, is a pivotal enzyme in the flavonoid biosynthetic pathway that plays a crucial role in producing simple and condensed anthocyanins. This enzyme catalyzes the production of flavan-3, 4-diols (leucoanthocyanidins) via the reduction of three colorless dihydroflavonols, i.e., dihydrokaempferol, dihydroquercetin, and dihydromyricetin. These compounds are also intermediates of flavonol biosynthesis, occurring through the flavonol synthase reaction. These leucoanthocyanidins are subsequently



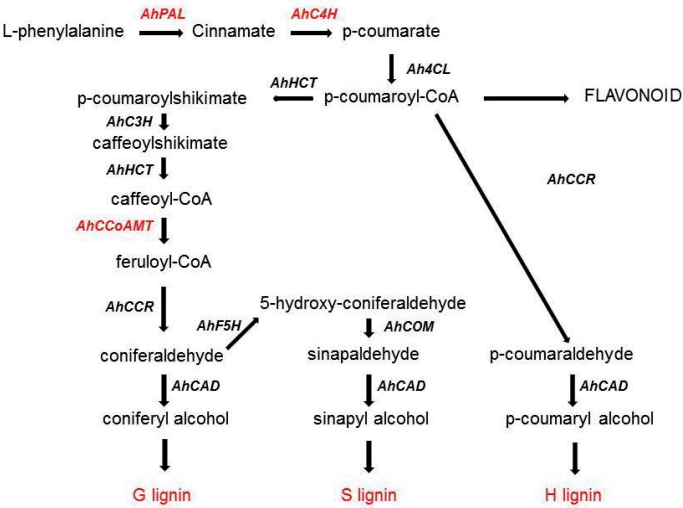
**Figure 6.** Flavonol biosynthesis in plants. The figure is modified from the original version in [136]. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; FLS, flavonol synthase. The genes in red text represent those found to be induced by stress conditions in the grain amaranth transcriptomic analysis [14].

converted to pelargonidin, cyanidin, and delphinidin, respectively. DFR can accept wide range of substrates, although substrate specificity of DFR has been shown to vary depending on the specific types of anthocyanins that accumulate in a given plant species. In plants, DFR can be either present as a single gene or as a multicopy gene family. Single DFR genes have been found in *Arabidopsis*, grape, tomato, rice, snapdragon, rose, barley, buckwheat, and grain amaranth (*AhDFR*) while multicopy DFR genes have been found in *Vitis vinifera*, *Ipomoea purpurea*, *Populus*, lotus, and *M. truncatula*. Increased flower pigmentation has been observed by transformation of petunia with a heterologous DFR. Also, the expression of *DFR* has been shown to be spatially and developmentally regulated, may be organ-specific, and its induction leads to the accumulation of anthocyanins in different plant tissues. Furthermore, tobacco plants overexpressing *CsDFR* showed early flowering and significantly higher seed yields, in addition to increased resistance to insect herbivory and antioxidant potential [165]. Other external factors can modulate the expression of *DFR*, including light and UV radiation, exogenous sucrose or JA, and cold or freezing stress [134,166].

Leucoanthocyanidin dioxygenase (*LDOX*: 1.14.11.19), also called 2-oxoglutarate iron-dependent dioxygenase (2-ODD) or anthocyanidin synthase (*ANS*), is also involved in anthocyanin biosynthesis and catalyzes the conversion of colorless to colored leucoanthocyanidin. Expression of *LDOX* has been detected in different organs of Shiraz grapevine, such as leaves, roots, seeds, flowers, berry skin, and flesh. An *LDOX* cDNA has been cloned from *Arabidopsis* and

the *transparent testa18* and *transparent testa19* mutants were subsequently shown to be *ldox* mutants. In developing *V. vinifera* grapes, the expression of *LDOX* was detected both before and after the ripening stage [134]. The detection of two *LDOX* genes in grain amaranth, one of which (*AhLDOX1*) was responsive to bacterial infection, was intriguing, considering the taxonomic restriction of anthocyanin synthesis in the Caryophyllales.

As mentioned, lignin is the generic term for a large group of aromatic polymers that result from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids (Figure 7). These polymers are deposited predominantly in the secondary walls of thickened cells, making them rigid and impermeable. In addition to developmentally programmed deposition of lignin, its biosynthesis can also be induced upon various biotic and abiotic stress conditions, such as wounding, pathogen infection, metabolic stress, and perturbations in cell wall structure. Activation of the monolignol precursor biosynthesis in the apoplast requires the combined activity of enzymes such as peroxidases (POX), laccases (LAC), or other polyphenol oxidases that transfer electrons from monolignols to electron receptors. These apoplastic enzymes interact with ROS such as hydrogen peroxide or superoxide, which act as electron receptors or modulators of POX and LAC enzymes through their signaling function. Once oxidized, monolignol radicals can bind to other similarly formed radicals to form the three-dimensionally cross-linked structures that characterize lignin. This polymerization process constitutes the final step of lignin biosynthesis.



**Figure 7.** The main biosynthetic route toward the monolignols p-coumaryl, coniferyl, and sinapyl alcohol (the figure is modified from the original version [66]. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; C3H, p-coumarate- 3-hydroxylase; HCT, p-hydroxycinnamoyl-CoA: quinate/shikimate p-hydroxycinnamoyltransferase; CCoAMT, caffeoyl-CoA o-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid O-ethyltransferase; CAD, cinnamyl alcohol dehydrogenase. The genes in red text represent those found to be induced by stress conditions in the grain amaranth transcriptomic analysis [14].



Contrary to lignin, lignans represent a structurally diverse class of plant-specialized metabolites that are ubiquitously distributed in all land plants. They are presumed to have a predominantly defensive role *in planta*, considering several reports that have described their antibacterial and/or antifungal activities. As such, lignans can act as both phytoanticipins and phytoalexins. In many tree species, the constitutive deposition of lignans in the heartwood is believed to reinforce durability, longevity, and resistance to many wood-rotting fungi. On the other hand, *de novo* formation of lignans in response to fungal attack has been reported in both woody and non-woody species [68]. Our transcriptomic-derived data indicate the possible presence of lignans biosynthetic genes in grain amaranth (Figure 8). This finding is in accordance with a recent report describing the deposition of these metabolites in seeds of *Amaranthus* species. The presence of lignans in amaranth seeds has been linked with some of its medicinal properties, considering the presumed antioxidant, antiviral, antitumoral, antibacterial, antifungal, insecticidal, estrogenic, and anti-estrogenic properties assigned to these particular secondary metabolites [3]. The flux of carbon to lignin biosynthesis can be seriously affected by changes in the expression levels of diverse other genes. Examples of a number of these genes, together with their respective homologs identified in grain amaranth, is described in Table 5.



**Figure 8.** Lignan biosynthetic pathway. DP, dirigent protein; PLR, pinoresinol/lariciresinol reductase; SIRD, secoisolariciresinol dehydrogenase. Two lignan-biosynthesis-related genes were found in grain amaranth; two of them code for DP and one for PLR.

Coumarins may be subclassified as simple coumarins (benzo- $\alpha$ -pyrones syn 1, 2-benzopyrone), 7-oxygenated coumarins (furanocoumarins syn. furobenzo- $\alpha$ -pyrones or furocoumarins) or pyranocoumarins (benzodipyran-2-ones). Simple coumarins, furanocoumarins, and pyranocoumarins share the same biosynthetic pathway, whereas the most common phenylcoumarins (i.e., coumestans) originate from isoflavone. Coumarin is characterized for its pleasant vanilla-like odor. The presence of this metabolite has been reported in a diversity of plants, including members of the Fabaceae, Lauraceae, Lamiaceae, Apiaceae, Asteraceae, Rutaceae, and Amaranthaceae (*Spinoside*, isoflavone). However, the transcriptomic data yielded no gene related to the biosynthesis of these interesting compounds, probably because they were not inducible under the stress conditions employed. Nevertheless, search for these genes in amaranth should continue due to the great interest invested in coumarins due to their potent physiological, bacteriostatic, and anti-tumor activity [167, 168]. Moreover, it has been proposed that coumarins may be engineered to have novel therapeutic properties by further derivatization of their backbone structure.

Gene	<sup>1</sup> No. Seq.	Function in stress	Reference(s)
Cinnamoyl-CoA-reductase ( <i>AhCCR</i> )	1	In wheat, this gene is involved in pathogen defense signaling. In tomato and other plants, the repression of this gene produced severe development-related phenotypes.	[189,190]
Cinnamyl alcohol dehydrogenase ( <i>AhCAD</i> )	16	The silencing <i>CAD</i> led to changes in the lignin structure, fiber quality, and/or improved digestibility. It also led to development related phenotypes during vegetative and reproductive stages. Pathogen resistance was also modified. Phylogenetic analysis suggests that the <i>AhCAD2</i> gene could be involved in stress tolerance responses.	[189,191,192]
Caffeoyl CoA 3-O-methyltransferase ( <i>AhCCoAOMT</i> )	3	This gene can be highly induced by drought and cold stress, suggesting a possible role in plant abiotic stress resistance. In maize and wheat, <i>CCoAOMT</i> is involved in the mechanism of resistance/susceptibility against fusariosis and powdery mildew, respectively.	[193–195]
4-Coumaroyl shikimate 3-hydroxylase/ p-coumarate 3-hydroxylase ( <i>AhC3H</i> )	1	C3H downregulation led to a dramatic accumulation of several glucosides of p-coumaric acid, including phenylglucosides (populoside, grandidentatin, and trichocarposide). This modification may be an acceptable strategy to increase the secondary metabolite diversity against pathogens.	[196]
Caffeic acid O-methyltransferase ( <i>AhCOMT</i> )	3	In wheat, silencing of this gene is linked to increased susceptibility to fungal pathogens, such as <i>Blumeria graminis</i> . Conversely, it led to an augmented resistance to <i>Hyaloperonospora arabidopsidis</i> in Arabidopsis.	[193,197]
Ferulic acid hydroxylase ( <i>AhF5H</i> )	2	<i>F5H</i> transcripts accumulated in the leaves in response to mechanical wounding or the application of related elicitors, such as ethylene, ABA, and hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> ). <i>f5h</i> mutants were unable to develop normally under UV light, probably because of a lack of UV-radiation protective synapate esters. This gene is essential for expression of anthocyanin biosynthesis-associated genes and anthocyanin accumulation under photo-oxidative stress.	[198–201]
Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase ( <i>AhHCT</i> ).	2	Downregulation of the HCT gene in alfalfa plants exhibited constitutive activation of defense responses, triggered by release of bioactive cell wall fragments and production of hydrogen peroxide generated as a result of impaired secondary cell wall integrity.	[202]

<sup>1</sup> Number of genes detected in the grain amaranth transcriptome [14].

**Table 5.** Lignin biosynthetic pathway genes identified in grain amaranth

Finally, many stress-induced phenylpropanoids are classified as phytoalexins. These are antimicrobial compounds synthesized in response to pathogen attack. They include pterocarpans (e.g., glyceollin), isoflavans, prenylated isoflavonoids (e.g., kievitone), stilbenes, psoralens, coumarins, 3-deoxyanthocyanidins, flavonols (e.g., quercetin, kaempferol), and aurones. Most of these compounds have trivial names, such as the coumarins umbelliferone (7-hydroxycoumarin), esculetin (6, 7-dihydroxycoumarin), scopoletin (6-methoxy-7-hydroxycoumarin), and others. No phenylpropanoid phytoalexins have been reported in amaranth. Findings from a recent report [15] suggested, however, that they could be participating as part of the induced defense responses against bacterial pathogens produced by the application of defense-related inducers, such as BTH, JA, or the exposure to avirulent pathogens. This proposal is supported by the induced expression of *AhPAL* in addition to several other known defensive genes, including those coding for ROS-detoxifying enzymes and pathogenesis-related proteins.

## 4. Conclusion

The several hypotheses raised by the discovery of the numerous stress-related phenylpropanoid genes in grain amaranth represents a strong incentive for the initiation and subsequent deepening of secondary metabolite studies in *Amaranthus* plants, which may yield promising results in various areas of interest, including food science and nutrition, medicine and stress plant physiology, among others.

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## Tolerance of Plants to Toxicity Induced by Micronutrients

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Additional information is available at the end of the chapter

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### Abstract

Micronutrient elements such as zinc, boron copper, iron, manganese, molybdenum, and chlorine are frequently responsible by the regulatory activity of the cell organelles, being nutrients that are absorbed and found in lower concentrations in plant tissues, they also contribute to supply the nutritional exigency of the plant. Study with *Zea mays* plants exposed to Zn toxicity + Si presented significant increases in stomatal conductance, net photosynthetic rate, transpiration rate, and water use efficiency, respectively, in comparison with treatment only with Zn. In relation to chlorophylls a, b and total and carotenoids presented non-significant increases, when compared to plants exposed to Zn toxicity. This study revealed the positive contribution of the Si on gas exchange and reduction of the negative effects provoked on chlorophylls and carotenoids in maize plants under Zn toxicity. Other results described that prolonged exposure to excessive Cu resulted in serious toxic effects on the rice seedlings. In contrast, Tre pretreatment has been shown to be beneficial in alleviating Cu toxicity, which was mainly attributed to the ability of Tre to restrict Cu uptake and accumulation to maintain Cu homeostasis, and to induce production of antioxidant and Gly enzymes to alleviate excessive Cu-triggered oxidative stress. Stress caused by the excessive supply of micronutrients to plants frequently promotes repercussions on oxidant system, inducing the overproduction of reactive oxygen species. The oxidative damage is a situation characterized by the large ROS accumulation and insufficient detoxification promoted by antioxidant enzymes, such as catalase and glutathione peroxidase. Different mechanisms have been proposed to explain the tolerance of plants to toxicity induced by micronutrients, as uses of other elements and substances, in which it can positively act with specific transporters, metal ion homeostasis and compartmentalization of micronutrients into the vacuole.

**Keywords:** gas exchange, antioxidant metabolism, metals, photosynthetic pigments

## **1. Introduction**

Large amounts of minerals and organic materials can be found in soil [1]; and some of these materials have great significance in the anatomical development and plant morphology. The soil fertility is an important factor of agronomic order. It is related to provision of essential elements in the soil, where the plant will absorb adequate levels of important substances to work with its metabolism [2]. The nutrients are arranged in ionic form, which can interact with the root system of plants via three mechanisms: interception root, diffusion, or mass flow [3].

The plants require basic conditions to develop and supply the essential elements to aid with the metabolism and produce simple and/or complex compounds [4]. These are divided into non-minerals and minerals, which are sub-divided into macro and micronutrients.

## **2. Non-mineral nutrients**

The non-mineral nutrients are hydrogen (H), oxygen (O), and carbon (C). These elements help in the formation of tissues. They are responsible for the composition of lipids, carbohydrates, proteins, nucleic acids, and hormones in plants [5]. Hydrogen (H) is the element that contributes to nitrogen fixation and photosynthesis; this is due to their reducing capacity molecules, being a nutrient assimilated from the water. Oxygen (O) is extracted from atmosphere and water, it is used during the process of cell respiration, where sugar transferred by the photosynthetic process is transformed into energy. The plant captures the Carbon (C) in the form of CO<sub>2</sub> from atmosphere. It is an organic nutrient with function to ensure the occurrence of physical, chemical, and biological cycles, ensuring the maintenance of energy reserves throughout vegetative growth.

## **3. Nutrients minerals**

These are elements essential for plant to complete its cycle [6]. They are divided into macro and micronutrients [7].

### **3.1. Macronutrients**

The macronutrients are composed by nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S). Large amount of these elements are required for plants to develop and meet its physiological activity. The macronutrients play a vital role in plant structure [8].

### **3.2. Micronutrients**

These elements are responsible for the regulatory activity of the cell organelles. These nutrients are absorbed and found in lower concentrations in plant tissues and supply the nutritional exigency of the plant [9].

#### 3.2.1. Zinc (Zn)

Zinc is an essential component in thousands of proteins in plants, in which it performs the role of enzymatic activator in various processes related to plant metabolism, such as dehydrogenases, aldolases, isomerases and transphorylases [10]. Its deficiency affects plant development, with stems and leaf expansion directly affected, and also reported to have consequences on chlorophyll levels and possibly necrosis [11].

#### 3.2.2. Boron (B)

Boron is a nutrient that works with the carbohydrate metabolism and transport of sugars through membranes. This element acts in the formation of the cell wall, sap movement, cell division, development of leaves and inflorescence, and confer resistance to tissues [12]. Its deficiency causes disorder in meristematic activities and reserve mechanisms can be directly affected, with the stem apex, flowers and fruits suffer deformations [13].

#### 3.2.3. Copper (Cu)

The copper activity is related to photosynthetic and respiration processes, fixation, and distribution of the nitrogen, all being necessary during plant cycle [14]. When the plant does not absorb adequate amount of this element for their development, it may present negative characteristics, such as changes of colour and structure in leaf [15].

#### 3.2.4. Iron (Fe)

Iron is an essential micronutrient used in photosynthesis, cell division, nitrogen fixation, and formation and morphology of plants [16-17]. The excess of this element affects the leaf, and their growth is strongly reduced [18]. The deficiency induces chlorosis [19].

#### 3.2.5. Manganese (Mn)

Manganese is a micronutrient of essential character. It plays the metabolic activities of direct or indirect orders, with capacity to activate enzymes responsible for the control of oxidation-reduction processes, such as the production of chlorophylls [20]. The low concentration of this micronutrient causes loss of leaf due to appearance of small spots on leaves and chlorosis, and structural development of the plant normally is compromised [21].

#### 3.2.6. Molybdenum (Mo)

Molybdenum is a micronutrient that helps in nitrogen fixation and nitrate assimilation [22]. With the deficiency of this nutrient, plants are affected by chlorotic spots and marginal necrosis, thus the plant yield is negatively affected [23].

#### 3.2.7. Chlorine (Cl)

Chlorine acts in the photosynthetic process of plants, aside from interacting with water and during transpiration process [24]. The lack of chlorine causes chlorosis and necrosis, roots

suffer atrophy, affecting the development of the plant [25]. The excess of chlorine in the soil is more common than the deficiency. Indication of its excess is signalled by the burn of the leaf edges [26].

#### 4. Toxicity of micronutrients

Despite micronutrients be required in higher plants, in higher concentrations frequently is toxic and provokes negative effects [27], as reduction in photosynthetic pigments [28], minor integrity and permeability of membranes [29], increase of the oxidative stress related with production and accumulation of reactive oxygen species (ROS), besides to increase the activities of antioxidant enzymes [30], and in levels more extremes to induce cell death [31].

Stress caused by the excessive supply of nutrients to plants promotes repercussion on oxidant system [32-33], inducing the overproduction of reactive oxygen species (ROS) as superoxide radical ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) [34]. The oxidative damage is a situation characterized by the large ROS accumulation and insufficient detoxification promoted by antioxidant enzymes, such as catalase and glutathione peroxidase [35].

Different mechanisms have been proposed to explain the tolerance of plants to toxicity induced by heavy metals and nutrients. Two specific transporters are metal ion homeostasis and compartmentalization of metals into the vacuole [36-37]. However, responses linked to contribution of Si in plants submitted to Zn excess, more specifically on gas exchanges and photosynthetic pigments, are unknown.

Beneficial repercussions related to Si uses in higher plants are intensively found [38-40]. Isa et al. [41] reported that Si is largely accumulated in leaves. Silva et al. [42] described increases in chlorophylls produced by exogenous Si application. Si also induces higher mechanical resistance from cell wall [43]. Chen et al. [44] found better light reception and increasing net photosynthesis rate and  $CO_2$  capitation after Si treatment.

#### 5. Objectives

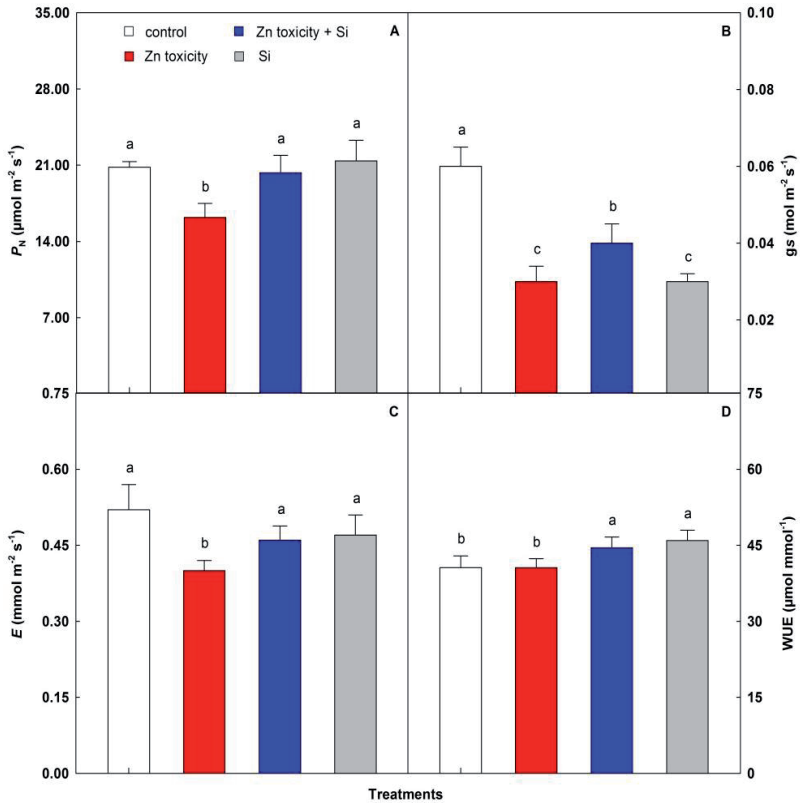
This chapter aim to: (i) define what nutrient toxicity is; (ii) present the modifications produced in the biochemical and physiological levels; (iii) explain the consequences to plant induced by the nutrient toxicity.

#### 6. Effects of zinc toxicity in gas exchange

Study conducted by Paula et al. [45] with *Zea mays* plants under Zn toxicity and treated with Si (silicon) evaluating the gas exchanges and photosynthetic pigments was shown in Figure 1. Zinc toxicity produced a negative interaction promoting stomatal closing, and consequently,



reduction in stomatal conductance. This effect can be attributed to Zn excess, in which it will induce a minor density and size of these structures, with structural differences in adaxial and abaxial sides, besides minor stomatal aperture sizes [46]. Similar results were obtained by Pavlíková et al. [47] in *Nicotiana tabacum* plants submitted to stress by Zn.



**Figure 1.** Stomatal Conductance (A), Photosynthesis Rate (B), Transpiration Rate (C), and Water Use Efficiency (D) in *Zea mays* plants subjected to silicon and zinc toxicity. Different letters to treatments indicate significant differences from the Skott-Knott test ( $P < 0.05$ ). Columns represent the mean values from four repetitions, and bars represent the standard deviations [45].

Zn induced a reduction in net photosynthetic rate, as explained by the stomatal limitation, arising of minor stomatal conductance, and consequent decrease of the  $\text{CO}_2$  assimilation to photosynthetic process [48-49]. Similar results were found by Shi and Cai [50] working with *Arachis hypogaea* plants submitted to Zn stress, corroborating with results obtained in this research.

The reduction of the transpiration in plants under exogenous application of Zn was possibly attributed to decrease in stomatal conductance. This stomatal limitation reduces the transpiration rate, promoting minor water loss from plant to atmosphere, and consequently limited nutrients reposition, in form of adsorbed ions into substrate with water, using the via root system [51]. In other words, the transpiration is responsible with the dynamic of nutrient transport from substrate in direction root and leaf [52], thus avoid the cavitation in xylem [53]. Fernández et al. It was also described that there is a significant reduction in transpiration rate in *Populus deltoides* plants submitted to high Zn concentrations [54].

The exogenous application of Si promoted an increase in water use efficiency (WUE), this result can be explained by the increase in net photosynthetic rate (PN) and maintenance in transpiration rate (E). The ratio between photosynthesis and transpiration will result in WUE [55], being a physiological parameter that describes quantitatively the behavior momentaneous of the gas exchanges in leaf, it also reveals the efficiency that the plant utilizes the water resource [56]. Our results are corroborated by Moussa [57] working with *Zea mays* seedlings under exogenous application of Si.

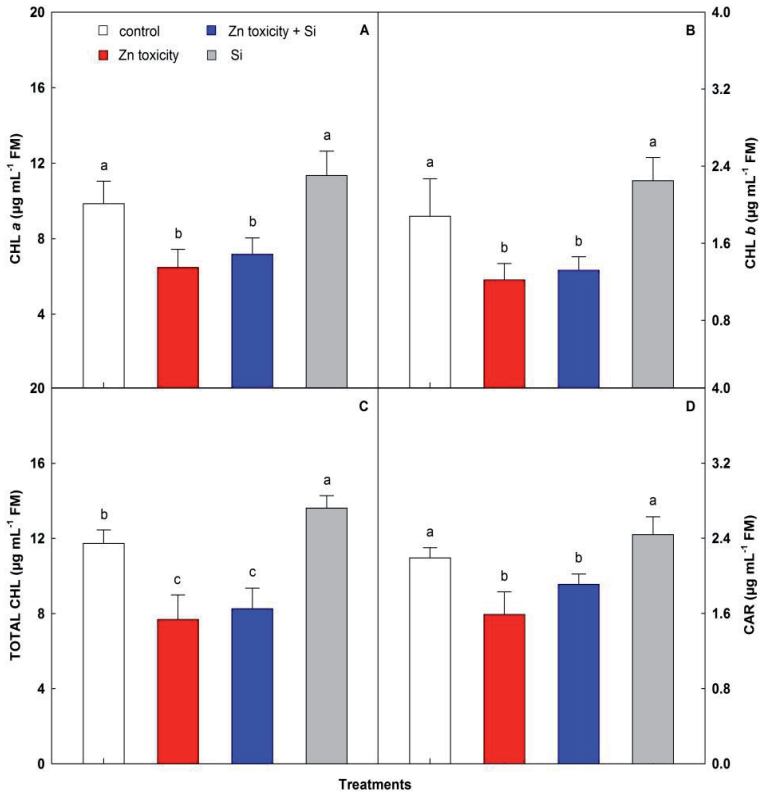
## 7. Zinc toxicity and silicon benefits on photosynthetic pigments

Figure 2 shows the same study conducted by Paula et al. [45] on photosynthetic pigments in *Zea mays* plants under Zn toxicity and Si. The decrease in CHL *a* occurred due to Zn toxicity, being probably related to minor biosynthesis rate of CHL *a* [58]. The Zn excess negatively interferes in NADPH availability into chloroplasts [59], because the NADPH is one of the substrates of the divinyl chlorophyllide an 8-vinyl-reductase enzyme, which is responsible to CHL *a* synthesis [60].

The chlorophylls are responsible to the photochemical and biochemical reactions during light capitation [61], while carotenoids present an important role related to photoprotection against excessive sunlight [62], given that both pigments work simultaneously into photosynthetic machinery [63]. However, the excessive B supply represents a problem to photosynthetic pigments, with consequent decrease in chlorophylls [64-65].

The Zn excess promoted a decrease in CHL *b* level, and this result can be explained by the oxidative stress induced by the overproduction of reactive species oxygen (ROS), such as H<sub>2</sub>O<sub>2</sub> [66-67]. The H<sub>2</sub>O<sub>2</sub> aside from being toxic in chloroplasts, is considered an inhibitor of the carbon metabolism, [68], resulting in acceleration of leaf senescence through of the lipid peroxidation and oxidative damages [69]. Similar results were reported by Bettaieb [70] evaluating *Solanum tuberosum* cultivars.

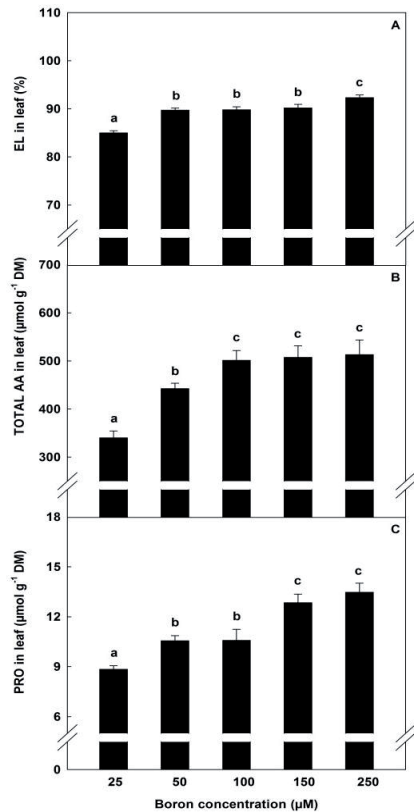
The CHL total levels were reduced after Zn toxicity, which is related to magnesium (Mg) substitution in molecule of chlorophyll by the Zn. It will result to the inadequate work of the light-harvesting complex (LHCII), and consequently the photosynthesis limitation [71-73]. Our results on reduction in CHL total were corroborated by Bassi and Sarma [74] in *Triticum aestivum* seedlings.



**Figure 2.** Chlorophyll *a* (A), Chlorophyll *b* (B), Total Chlorophyll (C) and Carotenoids (D) in *Zea mays* plants subjected to silicon and zinc toxicity. Different letters to treatments indicate significant differences from the Skott-Knott test ( $P < 0.05$ ). Columns represent the mean values from four repetitions, and bars represent the standard deviations [45].

## 8. Boron toxicity induces modifications in nitrogen metabolism

Silva et al. [75] working with *Schizolobium parahyba* plants exposed to progressive boron levels determined amino acids total and proline is depicted in Figure 2. The total amino acids accumulated in leaf must be associated to proteases enzyme activity and concomitantly to inhibition of the protein biosynthesis rate, due to the high B levels. According to Paula et al. [76], the increases in proteases activities promote the breakdown and consequent decrease of proteins, increasing the total AA amount, which will be used in synthesis of other AA, like as PRO. Cervilla et al. [77] evaluating the effects of the B toxicity in *Solanum lycopersicum* plants found similar results.



**Figure 3.** Electrolyte Leakage (A), Total Amino Acids (B), Proline (C) of young *Schizolobium parahyba* var. *amazonicum* plants subjected to boron toxicity. Different letters for boron levels indicate significant differences from the Skott-Knott test ( $P<0.05$ ). Columns represent the mean values from 5 repetitions, and bars represent the standard deviations [75].

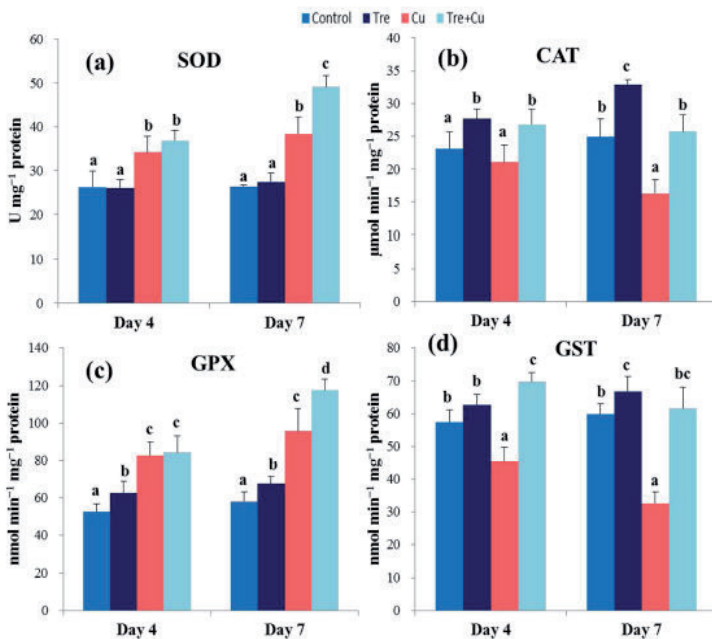
The proline concentration was maximized and this result is related to the amino acid acting in detoxification process of ROS and membrane protection against lipid peroxidation [78-79]; associated with ROS antagonist, it aims to attenuate the oxidative stress and to avoid the cell death. In other activities, the PRO protects the protein structure against denaturation and it will stabilize the cell membranes during interaction with phospholipids [80]. Contreras et al. [81] evaluating the B and NaCl effects in *Solanum lycopersicum* plants verified similar results of this research.

Proline is an amino acid synthetized into nitrogen metabolism with functions related to osmoprotection [82] and cellular homeostasis [83], which can contribute to improve the plant tolerance under situations of abiotic stress, as B toxicity.

## 9. Effects of the copper toxicity and exogenous trehalose on antioxidant enzymes

In agreement with Mostofa et al. [84], the regulating Cu homeostasis is crucial in maintaining the intracellular Cu level to avoid toxicity. Plants have developed various mechanisms to restrict Cu toxicity, such as inhibition of Cu uptake by binding with root exudates like organic acids, intracellular sequestration by strong ligands like cysteine-rich compounds and phytochelatins, and exclusion of excessive Cu from the cells by sugar alcohols like trehalose (Tre) [26–28]. Tre, a non-reducing disaccharide of glucose, protects plant cells against long-term desiccation by stabilizing enzymes, proteins, and biological membranes under dehydration [29].

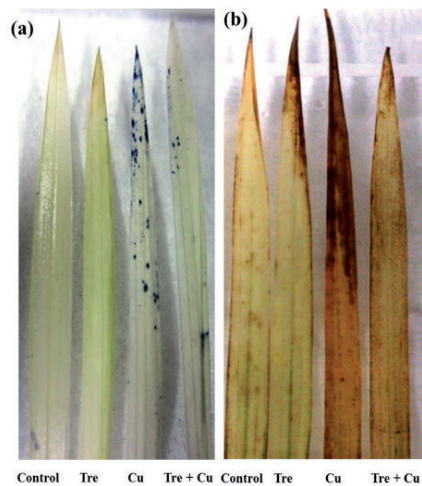
In relation to antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GST), the pretreatment with non-reducing disaccharide Tre induces increases in enzyme activities when compared with control or Cu stress treatments (Figure 4).



**Figure 4.** Effect of exogenous trehalose on the activities of antioxidant enzymes in rice seedlings with or without Cu stress. (a) Superoxide Dismutase (SOD), (b) Catalase (CAT), (c) Glutathione Peroxidase (GPX), and (d) Glutathione Reductase (GST). Control, Tre, Cu, and Tre + Cu correspond to control, 10 mM trehalose, 100 μM CuSO<sub>4</sub>, and 10 mM trehalose + 100 μM CuSO<sub>4</sub>, respectively. Bars represent standard deviation (SD) of the mean (n = 3). Different letters indicate significant differences among treatments at p < 0.05, according to Duncan's multiple range test [84].

NBT staining indicated an increased amount of  $O_2^-$  as scattered dark blue spots in the leaf plate of the Cu-stressed seedlings compared with the non-treated control (Figure 5a). Similarly, DAB staining confirmed a marked increase in brown polymerization products, which indicated the over-accumulation of  $H_2O_2$  in the leaves of the Cu-stressed seedlings relative to control (Figure 5b) [84].

Results described by Mostofa et al. [84] indicate that prolonged exposure to excessive Cu resulted in serious toxic effects on the rice seedlings. In contrast, Tre pretreatment has been shown to be beneficial in alleviating Cu toxicity, which was mainly attributed to the ability of Tre (i) to restrict Cu uptake and accumulation to maintain Cu homeostasis, and (ii) to induce production of antioxidant and Gly enzymes to alleviate excessive Cu-triggered oxidative stress [84].



**Figure 5.** Effect of exogenous trehalose on ROS accumulation in leaves of rice seedlings with or without Cu stress. (a) Superoxide ( $O_2^-$ ) and (b) Hydrogen Peroxide ( $H_2O_2$ ) production in rice leaves were detected using nitro-blue tetrazolium (NBT) solution and 3,3'-diaminobenzidine (DAB), respectively, at day 7 of Cu stress. Control, Tre, Cu, and Tre + Cu correspond to control, 10 mM trehalose, 100  $\mu$ M  $CuSO_4$ , and 10 mM trehalose + 100  $\mu$ M  $CuSO_4$ , respectively [84].

## 10. Final considerations

Micronutrient elements such as zinc, boron copper, iron, manganese, molybdenum, and chlorine are responsible to the regulatory activity of the cell organelles. These nutrients are absorbed and found in lower concentrations in plant tissues, and contribute to supply the nutritional exigency of the plant.

Study with *Zea mays* plants exposed to Zn toxicity + Si presented more significant increases in stomatal conductance, net photosynthetic rate, transpiration rate, and water use efficiency

compared with the treatment only with Zn. On the contrary, chlorophylls a, b and total and carotenoids presented non-significant increases when compared with plants exposed to Zn toxicity.

This study also revealed the positive contribution of the Si on gas exchange and reduction of the negative effects provoked on chlorophylls and carotenoids in *Zea mays* plants under Zn toxicity. Other result described that prolonged exposure to excessive Cu resulted in serious toxic effects on the rice seedlings. In contrast, Tre pretreatment has been shown to be beneficial in alleviating Cu toxicity, which was mainly attributed to the ability of Tre to restrict Cu uptake and accumulation to maintain Cu homeostasis. It induced production of antioxidant and Gly enzymes to alleviate excessive Cu-triggered oxidative stress.

Stress caused by the excessive supply of micronutrients to plants promotes repercussion on oxidant system, inducing the overproduction of reactive oxygen species. The oxidative damage is a situation characterized by the large ROS accumulation and insufficient detoxification promoted by antioxidant enzymes, such as catalase and glutathione peroxidase. Different mechanisms have been proposed to explain the tolerance of plants to toxicity induced by micronutrients, as uses of other elements and substances, in which it can positively act with specific transporters, metal ion homeostasis and compartmentalization of micronutrients into the vacuole.

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# Abiotic and Biotic Elicitors–Role in Secondary Metabolites Production through In Vitro Culture of Medicinal Plants

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Additional information is available at the end of the chapter

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## Abstract

Plant secondary metabolites are having the great application in human health and nutritional aspect. Plant cell and organ culture systems are feasible option for the production of secondary metabolites that are of commercial importance in pharmaceuticals, food additives, flavors, and other industrial materials. The stress, including various elicitors or signal molecules, often induces the secondary metabolite production in the plant tissue culture system. The recent developments in elicitation of plant tissue culture have opened a new avenue for the production of secondary metabolite compounds. Secondary metabolite synthesis and accumulation in cell and organ cultures can be triggered by the application of elicitors to the culture medium. Elicitors are the chemical compounds from abiotic and biotic sources that can stimulate stress responses in plants, leading to the enhanced synthesis and accumulation of secondary metabolites or the induction of novel secondary metabolites. Elicitor type, dose, and treatment schedule are major factors determining the effects on the secondary metabolite production. The number of parameters, such as elicitor concentrations, duration of exposure, cell line, nutrient composition, and age or stage of the culture, is also important factors influencing the successful production of biomass and secondary metabolite accumulation. This chapter reviews the various abiotic and biotic elicitors applied to cultural system and their stimulating effects on the accumulation of secondary metabolites.

**Keywords:** Cell culture, elicitor, organ culture, secondary metabolites, stress

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## 1. Introduction

The total mankind is dependent on plants as a source of carbohydrates, proteins, vitamins, food, and shelter. Plants are studied for their important constituents and the nutritional factors

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for over decades. Along with the essential primary metabolites, higher plants are also capable to produce a number of low molecular weight compounds. A diverse group of organic compounds that are produced by plants to facilitate interaction with the biotic environment and the establishment of a defense mechanism are called as plant secondary metabolites [1–3]. The production of these metabolites is very low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant [4,5]. Plant natural products have been an important part of medicine throughout human history. In recent years, the use of herbal medicines has steadily increased worldwide [6]. With this increasing demand comes growing concerns about the safety and efficacy of herbal medicines. Although the potential for medicinal plants seems almost limitless, there are a few major obstacles that hinder large-scale utilization by the western medical system. Among them is the lack of reproducibility common in testing many plant extracts (up to 40%), which has limited the enthusiasm for developing plant-based pharmaceuticals [7]. Unlike standardized single-entity pharmaceutical drugs, herbal medicines consist of complex mixtures with multiple compounds responsible for therapeutic activity, making standardization difficult [8]. Further complicating the issue is the fact that plants, unlike synthetic medicines, are living organisms, with inherent biological variation [9]. Just because plant material originates from the same species, it does not necessarily mean that the chemical content will be identical. This lack of reproducibility may be due to two main factors, genetic variability and differences in growing conditions.

In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives. Plants are producing new compounds and in future new chemical models are drawing for new drugs because the most of the plants chemistry is yet to be explored [10]. The characterization of molecular structures and chemical analysis helped us to pinpoint the activities of plants under controlled conditions. Although all these advancements, we still depend on the secondary metabolites of biological sources including pharmaceuticals [11].

Due to various agro alimentary, perfumes, flavors, colors, and pharmacological effects, the secondary metabolites are having extensive demand and various commercial preparations are available in the market. Besides, the appeal of using natural products for medicinal purposes is increasing, and metabolic engineering can alter the production of pharmaceuticals and help to design new therapies. The evolving commercial importance of secondary metabolites has in recent years resulted in a greater interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites [12]. Secondary metabolites are separated into nitrogen compounds (alkaloids, nonprotein amino acids, amines, alcamides, cyanogenic glycosides, and glucosinolates) and nonnitrogen compounds (monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes, saponins, flavonoids, steroids, and coumarins).

The plant tissue culture plays an important role in the rapid clonal propagation, regeneration of genetically manipulated superior clones, conservation of germplasm, production of secondary metabolites, and ex vitro conservation of valuable phytodiversity [13,14]. The plant,



cell, tissue and organ culture techniques have come up with an escapable tool with the possibilities of acclaiming and supplementing the conventional method in plant breeding, plant improvement, and biosynthetic pathways. This technique has several potential applications in crop improvement, and efficient regeneration is a prerequisite in such improvement programs. The biotechnological production of secondary metabolites in plant cell and organ cultures is an attractive alternative to the extraction of the whole plant material [15]. In particular, plant-specific important compounds are obtained by using the plant cell and organ cultures [2]. The faster proliferation rates and shorter biosynthetic cycle of cell and organ cultures leads to have a higher rate of metabolism when compared to field grown plants [16]. Further, plant cell/organ cultures are under controlled conditions proliferates at their optimum growth rates when compared to the cultivated plants, which are facing environmental, ecological, and climatic variations. In recent years, various strategies have been developed for use in biomass accumulation and the synthesis of secondary compounds, such as strain improvement, optimization of medium, and culture environments, elicitation, precursor feeding, metabolic engineering, permeabilization, immobilization, and biotransformation methods, bioreactor cultures, and micropropagation [17]. The focus of the present chapter is the influence of abiotic and biotic elicitors on the secondary metabolite production in the *in vitro* cultured medicinal plants.

## **2. Classification of elicitors and secondary metabolite production via *in vitro* culture of medicinal plants**

Stress is an important factor in determining the chemical composition and therapeutic activity of medicinal plants. Actively stimulating, or eliciting, the plant stress response to induce the desired chemical response is called elicitation, harnessing the connection between plant stress and phytochemistry. "Elicitor may be defined as a substance for stress factors which, when applied in small quantity to a living system, it induces or improves the biosynthesis of specific compound which do have an important role in the adaptations of plants to a stressful conditions" [18]. Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors [18]. Several biotechnological strategies have been hypothesized and applied for the productivity enhancement, and elicitation is recognized as the most practically feasible strategy for increasing the production of desirable secondary compounds from cell, organ, and plant systems [19–21].

On the basis of nature, elicitors can be divided into two types abiotic and biotic (Figure 1). Abiotic elicitors comprise of substances that are of nonbiological origin and are grouped in physical, chemical, and hormonal factors. Biotic elicitors are the substances of biological origin that include polysaccharides originated from plant cell walls (e.g. chitin, pectin, and cellulose) and micro-organisms.

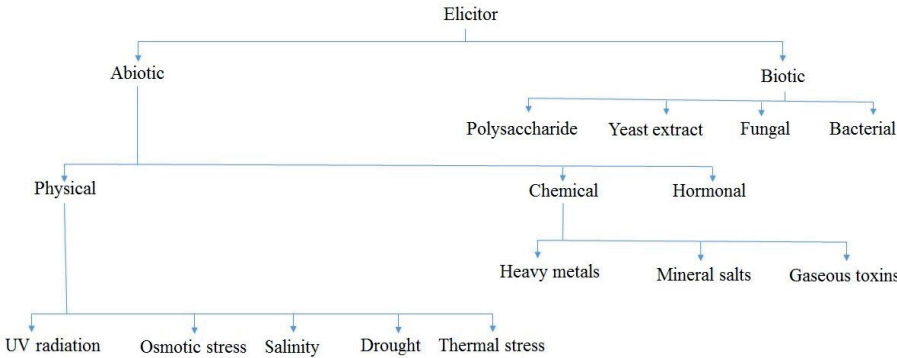


Figure 1. Elicitors Classification Based on their Nature

3. Abiotic elicitors

As mentioned above, the abiotic elicitors are categorized into physical, chemical, and hormonal elicitors. Abiotic elicitors have wide range of effects on the plants and in the production of secondary metabolites (Table 1).

Elicitor	Plant species	Nature of culture	Compounds	References
Ozone (O <sub>3</sub> )	<i>Melissa officinalis</i>	Shoot	Rosmarinic acid	[163]
	<i>Hypericum perforatum</i>	Cell suspension	Hypericin	[164]
	<i>Pueraria thomsnii</i>	Cell suspension	Puerarin	[165]
pH	<i>Bacopa monnieri</i>	Shoot	Bacoside A	[166]
	<i>Withania somnifera</i>	Hairy root	Withanolide A	[167]
	<i>Withania somnifera</i>	Cell suspension	Withanolide A	[168]
Sucrose	<i>Hypericum adenotrichum</i>	Seedling	Hypericin and pseudohypericin	[41]
	<i>Corylus avellana</i>	Cell suspension	Paclitaxel	[169]
	<i>Bacopa monnieri</i>	Shoot	Bacoside A	[166]
	<i>Withania somnifera</i>	Cell suspension	Withanolide A	[168]
Ultraviolet C	<i>Vitis vinifera</i>	Cell suspension	Stilbene	[34]
Proline	<i>Stevia rebaudiana</i>	Callus and suspension	Steviol glycoside	[40]
Polyethylene glycol	<i>Stevia rebaudiana</i>	Callus and suspension	Steviol glycoside	[40]

Elicitor	Plant species	Nature of culture	Compounds	References
Jasmonic acid	<i>Hypericum adenotrichum</i>	Seedling	Hypericin and pseudohypericin	[41]
	<i>Bacopa monnieri</i>	Shoot	Bacoside A	[170]
	<i>Plumbago indica</i>	Hairy root	Plumbagin	[88]
	<i>Plumbago rosea</i>	Cell suspension	Plumbagin	[86]
Methyl jasmonate	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinone	[94]
	<i>Perovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
	<i>Vitis vinifera</i>	Cell suspension	Stilbene	[34]
	<i>Bacopa monnieri</i>	Shoot	Bacoside	[96]
	<i>Salvia officinalis</i>	Shoot	Diterpenoid	[171]
	<i>Silybum marianum</i>	Cell suspension	Silymarin	[172]
	<i>Salvia castanea</i>	Hairy root	Tanshinone	[173]
	<i>Gymnema sylvestree</i>	Cell suspension	Gymnemic acid	[148]
	<i>Withania somnifera</i>	Hairy root	Withanolide A, withanone, and withaferin A	[95]
	<i>Andrographis paniculata</i>	Cell suspension	Andrographolide	[97]
	<i>Vitis vinifera</i>	Cell suspension	trans-Resveratrol	[91]
	<i>Taverniera cuneifolia</i>	Root	Glycyrrhizic acid	[135]
	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinones	[174]
Gibberelic acid	<i>Echinacea pupurea</i>	Hairy root	Caffeic acid derivatives	[175]
	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinone	[94]
Salicylic acid	<i>Vitis vinifera</i>	Cell suspension	Stilbene	[34]
	<i>Digitalis purpurea</i>	Shoot	Digitoxin	[176]
	<i>Hypericum hirsutum</i>	Shoot	Hypericin and pseudohypericin	[177]
	<i>Gymnema sylvestree</i>	Cell suspension	Gymnemic acid	[148]
	<i>Withania somnifera</i>	Hairy root	Withanolide A, withanone, and withaferin A	[95]
	<i>Datura metel</i>	Root	Hyoscyamine and scopolamine	[178]
	<i>Glycyrrhiza uralensis</i>	Adventitious root	Glycyrrhizic acid	[179]

Elicitor	Plant species	Nature of culture	Compounds	References
Sodium salicylate	<i>Salvia officinalis</i>	Shoot	Carnosol	[180]
Sodium chloride	<i>Catharanthus roseus</i>	Embryogenic tissues	Vinblastine and vincristine	[55]
Sorbitol	<i>Peroovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
Silver (Ag)	<i>Peroovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
	<i>Vitis vinifera</i>	Cell suspension	Resveratrol	[67]
	<i>Salvia castanea</i>	Hairy root	Tanshinone	[173]
	<i>Datura metel</i>	Hairy root	Atropine	[150]
Cadmium (Cd)	<i>Vitis vinifera</i>	Cell suspension	Resveratrol	[67]
	<i>Datura stramonium</i>	Root	Sesquiterpenoid	[79]
Cobalt (Co)	<i>Vitis vinifera</i>	Cell suspension	Resveratrol	[67]
Copper (Cu)	<i>Ammi majus</i>	Shoot	Xanthotoxin	[181]
	<i>Bacopa monnieri</i>	Shoot	Bacoside	[170]
	<i>Datura stramonium</i>	Root	Sesquiterpenoid	[79]

**Table 1.** Effect of Different Abiotic Elicitors on the Production of Various Secondary Metabolites in Plants

3.1. Physical elicitors

Physical elicitors include light, osmotic stress, salinity, drought, and thermal stress.

3.1.1. Light

The light is a physical factor that can affect the metabolite production. Light can stimulate such secondary metabolites include gingerol and zingiberene production in *Zingiber officinale* callus culture [22]. The effect of light irradiation on anthocyanin production in cell suspension cultures of *Perilla frutescens* was reported [23]. The effect of light and hormones on the digitoxin accumulation in *Digitalis purpurea* L. was reported by Hagimori et al. [24]. Moreover, in hairy root cultures of *Artemisia annua*, the effect of light irradiation influenced the artemisinin biosynthesis [25]. The effect of white light on taxol and baccatin III accumulation in cell cultures of *Taxus cuspidate* was reported by Fett-Neto et al. [26]. Ultraviolet (UV) radiation stimulates secondary metabolite production. Increasing UV-B exposure in field-grown plants not only increased the total essential oil and phenolic content but also decreased the amount of the possibly toxic beta-asarone [27]. These findings are to be expected as phenolics are known UV protectants [28]. *Catharanthus roseus* plants, exposed to UV-B light, show significant increases in the production of vinblastine and vincristine, which have proven effective in the treatment of leukemia and lymphoma [29]. UV-C irradiation promotes the phenylpropanoid pathway

and stimulates flavonoid synthesis [30]. UV-C irradiation is an effective method to enhance stilbene production in *Vitis vinifera* berries [31], *V. vinifera* leaves [32], and *V. vinifera* callus of different genotypes [33]. UV-C together with methyl jasmonate (MeJA) or salicylic acid (SA) also used to enhance stilbene production in *V. vinifera* cell cultures [34].

### 3.1.2. Osmotic stress

Osmotic stress (water stress) is an abiotic physical elicitor [35] and is one of the important environmental stresses that can alter the physiological and biochemical properties of plants and increase the concentration of secondary metabolites in plant tissues [36]. Proline acts as an osmolyte, as protective agent for cytoplasmic enzymes, as a reservoir of nitrogen and carbon sources for post-stress growth, or even as a stabilizer of the machinery for protein synthesis, regulation of cytosolic acidity and scavenging of free radicals [37]. However, the various roles of proline have been proposed, but the main role could be the osmotic adjustment in osmotically stressed plant tissues and the protection of plasma membrane integrity [38]. Polyethylene glycol (PEG) is an osmotic agent (nonpenetrating osmoticum) that has been used for induction of water stress in many plants [39]. The proline and PEG enhanced the production of steviol glycosides content in both callus as well as suspension culture of *Stevia rebaudiana* [40]. PEG elicited the pharmacologically active compounds, such as hypericin and pseudohypericin, in *Hypericum adenotrichum* [41]. Sucrose is a typical osmotic stress agent used for the induction of water stress in plants that also serves as a vital carbon and energy source [42]. It has been shown that water and osmotic imbalance can strongly influence the synthesis of hypericin and hyperforin in *Hypericum perforatum* plants [43]. In addition, it has been reported that both hypericin and pseudohypericin concentrations decreased, while hyperforin concentration increased significantly in the plants grown under water stress conditions [36].

### 3.1.3. Salinity

Salinity reduces plant growth and development and alters a wide array of physiological and metabolic processes [44,45]. Plants have developed complex mechanisms for adaptation to the osmotic, ionic, and oxidative stresses that are induced by the salt stress. Exposure to salinity is known to induce or stimulate the production of secondary plant products, such as phenols, terpenes, and alkaloids [46–48]. *C. roseus* grown under salt stress showed increased levels of the alkaloid vincristine [49]. In *Grevillea*, a significant increase in anthocyanin concentration was reported under salinity exposure in both the salt-tolerant *Grevillea ilicifolia* and the salt-sensitive *Grevillea arenaria* [50]. In contrast to this, salt stress decreased the anthocyanin level in the salt-sensitive species [51]. In *Datura innoxia*, salt treatment increased the total alkaloid content in young leaves, and the results indicated that at the organ level, tropane alkaloid accumulation was related to plant growth [52]. Glycine betaine was increased under salinity in numerous species including *Triticum aestivum* [53] and *Trifolium repens* [54]. Salinity also increased the diamine and polyamine content in *Oryza sativa* [53]. An improved synthesis of vinblastine and vincristine was observed in *C. roseus* embryogenic tissue culture by using NaCl as an elicitor [55].

### 3.1.4. Drought stress

One of the most important abiotic stress is drought, which affect plant growth and their developmental process [56]. The available water in the soil is reduced to such critical levels, and atmospheric conditions add to the continuous loss of water; the situation is called drought stress. The high temperature in the environment and solar radiations add up the water deficit in the soil, which leads to drought stress. Drought stress tolerance is observed in all types of plants, but its extent varies from species to species [56]. Drought stress, which can also greatly reduce plant growth, can increase secondary metabolite content. Mild water stress significantly increased the content of the anti-inflammatory saikosaponins in *Bupleurum chinense* [57]. Moderate water stress increased the content of salvianolic acid in roots of *Salvia miltiorrhiza*, although the content of other bioactives, including tanshinone, was lowered [58]. Moderate drought stress also increased the production of rosmarinic, ursolic, and oleanolic acid in *Prunella vulgaris* [59]. A weak water deficit greatly increased the glycyrrhizic acid content in roots of *Glycyrrhiza uralensis* [60]. In *Hypericum brasiliense*, the amounts of various phenols and betulinic acid were drastically increased under drought stress [61].

### 3.1.5. Thermal stress

Although thermal stress can greatly reduce plant growth and induce senescence, elevated temperatures (heat stress) or low temperatures (cold stress) have also been shown to increase secondary metabolite production. Temperature strongly influences metabolic activity and plant ontology, and high temperatures can induce premature leaf senescence [62]. Elevated temperatures increase leaf senescence and root secondary metabolite concentrations in the herb *Panax quinquefolius* [63]. A 5°C increase in temperature significantly increased the ginsenoside content in roots of *P. quinquefolius* [63]. A temperature variation has multiple effects on the metabolic regulation, permeability, and rate of intracellular reactions in plant cell cultures [62]. Temperature range of 17–25°C is normally used for the induction of callus tissues and growth of cultured cells [16]. The temperature and light quality influences on the production of ginsenoside in hairy root culture of *Panax ginseng* [64]. The *Melastoma malabathricum* cell cultures incubated at a lower temperature range ( $20 \pm 2^\circ\text{C}$ ) grew better and had higher anthocyanin production than those grown at  $26 \pm 2^\circ\text{C}$  and  $29 \pm 2^\circ\text{C}$  [65]. Fifteen days at 35°C significantly increased the hypericin and hyperforin content in shoots of *Hypericum perforatum* [66].

## 4. Chemical elicitors

Heavy metals have become one of the main abiotic stress agents for living organisms because of their increasing use in the developing fields of industry and agrotechnics and high bioaccumulation and toxicity [67]. Although a lot of information is available concerning the effects of heavy metals on plant growth and physiology, much less is known regarding their effects on the production of secondary metabolites. Heavy metal-induced

changes in metabolic activity of plants can affect the production of photosynthetic pigments, sugars, proteins, and nonprotein thiols. These effects can result from the inhibition of enzymes involved in the production of these natural products, likely through impaired substrate utilization [68]. Metals may alter the production of bioactive compounds by changing aspects of secondary metabolism [2]. Metals including Ni, Ag, Fe, and Co have been shown to elicit the production of secondary metabolites in a variety of plants [69].

An increased oil content up to 35% in *Brassica juncea* was seen due to the effective accumulation of metals (Cr, Fe, Zn, and Mn) [70]. The highest accumulations of secondary metabolites such as shikonin [71] and also the production of digitalin [72] were observed by treating Cu<sup>2+</sup> and Cd<sup>2+</sup>. The production of betalains in *Beta vulgaris* also stimulated by Cu<sup>2+</sup> [73]. Co<sup>2+</sup> and Cu<sup>2+</sup> have a stimulatory effect on the production of secondary metabolites in *Beta vulgaris* [73]. The betalains production was enhanced by exposing the hairy root culture to metal ions [74]. The stimulatory effects of Cu<sup>2+</sup> on the accumulation of betacyanins in callus cultures of *Amaranthus caudatus* were reported by Obrenovic [75]. The addition of Zn<sup>2+</sup> (900 µM) improved the yield of lepidine in cultures of *Lepidium sativum* [76]. However, Cu proved more effective than Zn in enhancing the yield product [76]. In hairy root cultures of *Brugmansia candida*, silver nitrate (AgNO<sub>3</sub>) or cadmium chloride (CdCl<sub>2</sub>) elicited the overproduction of two tropane alkaloids, scopolamine, and hyoscyamine [20]. The production of taxol in cell culture of *Taxus* sp. was enhanced by the rare-earth metal (lanthanum) [77]. AgNO<sub>3</sub> stimulated the production of tanshinone in the root culture of *Peroovskia abrotanoides* [78]. The treatment of root cultures of *Datura stramonium* with cadmium salts at external concentrations of approximately 1 mM has been found to induce the rapid accumulation of high levels of sesquiterpenoid-defensive compounds, notably lubimin and 3-hydroxylubimin, but not alkaloid [79].

## 5. Hormonal elicitors

Various plant hormones have been extensively used in elicitation studies. The most studied, because of their key roles in the plant defense response, are jasmonic acid (JA) and SA and its derivatives.

### 5.1. Jasmonates

Jasmonates, including JA and MeJA, are a family of cyclopentanone compounds that modulate a wide range of plant responses [80,81] and act as effective elicitors to enhance secondary metabolites in in vitro cultures. They constitute an important class of elicitors for many plant secondary metabolic pathways, which are typically manifested by the elicitation of secondary metabolite biosynthesis when plants face particular environmental stresses [82]. JA is an important signal molecule of plant in response to wound and pathogen attack [83]. JA and its more active derivative MeJA can induce the production of a wide range of plant secondary metabolites such as rosmarinic acid, terpenoid indole alkaloid, and plumbagin in various cell

cultures [84–86]. JA elicitation are reported to induce the production of rosmarinic acid in *Mentha piperita* [84], anthocyanin in *V. vinifera* [87], and plumbagin in hairy roots of *Plumbago indica* [88]. JA and MeJA have been used as elicitors for stilbene biosynthesis in *V. vinifera* foliar cuttings [89], *V. vinifera* cell cultures [90,91], and *Vitis rotundifolia* hairy root cultures [92]. The addition of MeJA to *V. vinifera* cell cultures also promoted anthocyanin accumulation [93]. MeJA with transgenic technology highly enhanced the production of tanshinones in *Salvia miltiorrhiza* hairy roots [94]. In the hairy root culture of *Withania somnifera*, MeJA elicited the production of withanolide A, withanone, and withaferin A [95]. The MeJA enhanced the production of bacoside A, a valuable triterpenoid saponin having nootropic therapeutic activity in in vitro shoot cultures of *Bacopa monnieri* [96]. In *Andrographis paniculata* cell culture, the MeJA induced the production of andrographolide content [97]. In *Glycyrrhiza glabra*, methyl jasmonate induced the production of the saponin soyasaponin [98]. It enhanced the production of paclitaxel in *Taxus canadensis* and *T. cuspidate* [99], and in *Rubus idaeus*, it stimulated the production of the raspberry ketone benzalacetone [100].

## 5.2. Salicylic acid

Salicylic acid, well known for the systemic acquired resistance it induces in the plant response to many pathogens, can also elicit the production of secondary metabolites in plants [101,102]. SA with transgenic technology highly enhanced the production of tanshinones in *S. miltiorrhiza* hairy roots [94]. The higher production of withanolide A, withanone, and withaferin A was reported in the elicited-hairy roots of *W. somnifera* [95]. SA induced the stilbene production in the cell suspension of *V. vinifera* [34]. It stimulated the production of alkaloids such as vincristine and vinblastine in periwinkle [103], the tropane alkaloid scopolamine in hairy root cultures of *Brugmansia candida* [77], and pilocarpine in jaborandi leaves [104]. Anthraquinone production was greatly increased in *Rubia cordifolia* after a SA treatment [105]. SA also affects terpenoid secondary metabolism in plants. It induced accumulation of the triterpenoids ginsenosides in ginseng and glycyrrhizin in licorice [106,107]. Recent evidence demonstrated that suitable concentrations of SA can also promote monoterpene production [108].

## 5.3. Gibberellic acid

Gibberellin (GA), a phytohormone, is also well known as an effective elicitor for the production of secondary metabolites [109].

# 6. Biotic elicitors

In the production of secondary metabolites from plants, the use of biotic elicitors had an important role (Table 2).



Elicitor	Plant species	Nature of culture	Compounds	References
Chitin	<i>Hypericum perforatum</i>	Shoot	Hypericin and pseudohypericin	[182]
	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[183]
	<i>Vitis vinifera</i>	Cell suspension	trans-Resveratrol and viniferins	[91]
Pectin	<i>Hypericum perforatum</i>	Shoot	Hypericin and pseudohypericin	[182]
Dextran	<i>Hypericum perforatum</i>	Shoot	Hypericin and pseudohypericin	[182]
Yeast extract	<i>Perovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
	<i>Plumbago rosea</i>	Cell suspension	Plumbagin	[86]
	<i>Silybum marianum</i>	Cell suspension	Silymarin	[172]
<i>Trichoderma atroviride</i>	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinone	[184]
<i>Protomyces gravidus</i>	<i>Ambrosia artemisiifolia</i>	Hairy root	Thiarubrine A	[134]
<i>Claviceps purpurea</i>	<i>Azadirachta indica</i>	Hairy root	Azadirachtin	[136]
<i>Mucor hiemalis</i>	<i>Taverniera cuneifolia</i>	Root	Glycyrrhizic acid	[135]
<i>Fusarium oxysporum</i>	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[183]
<i>Phoma exigua</i>	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[183]
<i>Botrytis cinerea</i>	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[183]
<i>Aspergillus niger</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[149]
<i>Saccharomyces cerevisiae</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[149]
<i>Agrobacterium rhizogenes</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[149]
<i>Bacillus subtilis</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[149]
<i>Escherichia coli</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[149]
	<i>Datura metel</i>	Hairy root	Atropine	[150]
<i>Bacillus cereus</i>	<i>Datura metel</i>	Hairy root	Atropine	[150]
<i>Staphylococcus aureus</i>	<i>Datura metel</i>	Hairy root	Atropine	[150]
<i>Rhizobium leguminosarum</i>	<i>Taverniera cuneifolia</i>	Root	Glycyrrhizic acid	[135]

**Table 2.** Effect of Different Biotic Elicitors on the Production of Various Secondary Metabolites in Plants

### 6.1. Polysaccharide

The biotic elicitors have been utilized to increase secondary metabolite production in medicinal plants. In a *Panax ginseng* cell suspension, the cell wall-derived elicitor oligogalacturonic acid significantly increased the ginseng saponin content [110]. The treatment of cultured cells of *Lithospermum erythrorhizon* with the polysaccharide agropectin induced the production of the naphthoquinone shikonin [111]. The chitosan treatment of cultures of *Plumbago rosea* increased the plumbagin content [112]. The application of chitin or chitosan induced the production of coumarins and fluoroquinolone alkaloids in shoot cultures of *Ruta graveolens* [113]. Chitosan enhanced the production of trans-resveratrol and viniferins in the cell system of *V. vinifera* [91]. Chitin induced the phenylpropanoid and naphthodianthrone production in cell suspension cultures of *H. perforatum* [114].

### 6.2. Yeast origin

For decades, scientists are using yeast extract as one of the biotic elicitors. Yeast extracts stimulated ethylene biosynthesis in tomato [115] and bacterial resistance in bean (*Phaseolus vulgaris*) [116]. Yeast extract elicited the production of tanshinone in the root culture of *Perovskia abrotanoides* [78].

### 6.3. Fungal origin

Biotic elicitors produced by pathogens have mainly been used to induce the plant defense response. In the past, biological mixtures were prepared from pathogens without identification of the active compounds. The use of pathogenic and nonpathogenic fungal preparations as elicitors has become one of the most effective strategies to induce phenylpropanoid/flavonoid biosynthetic pathways in plant cells [117,118]. Necrotrophic pathogens such as *Botrytis* sp. usually kill the host cells often through secretion of toxins before deriving nutrients from them [119]. On the other hand, biotrophic pathogens *Fusarium* sp. or *Phoma* sp. try to avoid killing the host cells, and derive their nutritional benefits from extensive contact with them and by altering the host metabolism and secretion systems [120,121]. An early defense reaction of the plant cell attacked by fungal pathogen includes the rapid and transient production of reactive oxygen species (ROS). Plant cells are usually protected against the detrimental effects of ROS by a complex of nonenzymatic and enzymatic antioxidant systems [122]. It has been demonstrated that the phenylalanine ammonia lyase (PAL) enzyme that catalyses the entry of L-phenylalanine into the phenylpropanoid pathway has reputedly a crucial role in the synthesis of antioxidant/defense-related compounds [117]. The mycelia extracts from the above mentioned fungi induced partitioning of the phenylpropanoid pathway and a rapid stimulation of the monolignol pathway in *Linum usitatissimum* cultured cells [123]. Cultures of *Phytophthora* elicited microbial resistance in soybean [124] and potato [125]. Extracts from microbial-enriched composts stimulated systemic resistance to *Phytophthora* in pepper (*Capsicum annuum*) [126]. As the plant defense response and the production of secondary metabolites are closely related, it is not surprising that a number of elicitors have also been shown to increase the production of secondary metabolites in medicinal plant cell culture. Similar to plant defense, initial work on secondary metabolite elicitation was performed using

biological mixtures. Fungal cell wall fragments increased the production of the indole alkaloids ajmalicine, serpentine, and catharanthine by up to five times in cell suspensions of *C. roseus* [69,127] and the 12-oxo-phytodienoic acid, raucaffricine, in *Rauwolfia canescens* [128]. Fungal mycelia increased the diosgenin content in *Dioscorea deltoidea* cells by 72% [129]. In *Papaver somniferum*, fungal spores increased the content of codeine, morphine, and sanguinarine by over eightfold [130,131]. A mixture of fungal polysaccharides increased the amount of the antimicrobial alkaloid acridone epoxide up to 100-fold in cultures of *R. graveolens* [132]. *Taxus chinensis* cells treated with an endophytic fungus found in the bark of the *T. chinensis* tree produced three times as much taxol as nonelicited cells [133].

The content of thiarubrine A was enhanced 3-fold in *Ambrosia artemisiifolia* hairy root cultures through the utilization of autoclaved cell wall filtrates from the fungus *Protomyces gravidus*, a pathogen of *Ambrosia artemisiifolia* [134]. The fungal challenged root cultures of *Taverniera cuneifolia*, increased the glycyrrhizic acid content [135]. Moreover, maximum increase in glycyrrhizic acid was noticed in *Mucor hiemalis* treated cultures. In case of *Fusarium moniliforme* and *Aspergillus niger*, threefold increase in glycyrrhizic acid was observed as compared to control unchallenged root culture. However, marginal increase in glycyrrhizic acid content was noticed, in *Penicillium fellutanum* and *Aspergillus tenuis* challenged cultures [135]. Similarly, biotic elicitors from *Claviceps purpurea* were included in *Azadirachta indica* hairy root cultures, leading to a 5-fold increase in the production of azadirachtin [136]. The transformed cell suspension cultures of *W. somnifera* when treated with the dual elicitation of copper sulfate (100  $\mu$ M) and the cell extract of *Verticillium dahliae* (5% v/v) showed highest production of withaferin A content when compared with the individual elicitors [137].

#### 6.4. Bacterial origin

The bacterial elicitors stimulated the biosynthesis of scopolamine in adventitious hairy root cultures of *Scopolia parviflora* via the inhibition of H6H (hyoscyamine 6 $\beta$ -hydroxylase) expression [138]. In bacterial elicitation, the maximum glycyrrhizic acid increase was observed in *Rhizobium leguminosarum* challenged culture as compared to unchallenged control roots of *Taverniera cuneifolia* [135]. Furthermore, in *Bacillus aminovorans*, *Agrobacterium rhizogenes*, and *Bacillus cereus* challenged cultures, significant increase in glycyrrhizic acid content was observed. However, root culture challenged by *Agrobacterium tumefaciens* did not show any significant increase in glycyrrhizic acid content [135]. The gradual increase in hypericin and pseudohypericin was observed in seedlings of *H. perforatum* after challenging with *Rhizobacterium* [139]. Coronatine, phytotoxin produced by the *Pseudomonas syringae* species significantly induced taxane synthesis in taxane media cell cultures [140], also induced the viniferins production in the cell culture of *V. vinifera* [91].

### 7. Parameters of elicitors

Elicitation has been widely used to increase the production or to induce de novo synthesis of secondary metabolites in in vitro plant cell cultures [141]. This opened up a new area of

research that could have important economic benefits for pharmaceutical industry. Several parameters such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line, growth regulation, nutrient composition, and quality of cell wall materials are also important factors influencing the successful production of secondary metabolite [142]. Some of these parameters were highlighted on elicitation of some medicinal plants for the production of secondary metabolites.

### 7.1. Elicitor concentration

Elicitor concentration plays a very important role in elicitation process. High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas an optimum level was required for induction [143–145]. At 0.1% (w/v) sodium chloride, ginseng saponin content and productivity were increased to approximately 1.15 and 1.13 times control values, respectively [146]. In the cell culture of *S. miltiorrhiza*, the effects of different concentrations of SA were affected the accumulation of salvianolic acid B and of caffeic acid. The increased accumulation of salvianolic acid B and of caffeic acid was observed in the applications of 3.125–25.0 mg/L of SA at 8 and 96 h when compared to the 32.0–50.0 mg/L of SA. After 96 h treatments with 3.125–25.0 mg/L of SA, the concentration of the phenolic acids decreased drastically compared to the amount 8 h after the treatments but still accumulated the higher concentrations of compound than that of the control [147]. The various concentrations (50, 100, 150, 200, and 250  $\mu$ M) of MeJA and SA were used in the cell suspension cultures of *Gymnema sylvestre*. The MeJA at 150  $\mu$ M and SA at 200  $\mu$ M enhanced the accumulation of gymnemic acid content [148]. In the hairy root culture of *W. somnifera*, the MeJA (15  $\mu$ M) and SA (150  $\mu$ M) enhanced the production of withanolide A, withanone, and withaferin A content [95]. In the cell suspension culture of *V. vinifera*, the cobalt at all three used concentrations (5.0, 25, and 50  $\mu$ M), Ag, and Cd at low concentration (5.0  $\mu$ M) were most effective to stimulate the phenolic acid production, increasing the 3-O-glucosyl-resveratrol up to 1.6-fold of the control level (250.5 versus 152.4  $\mu$ mol/g), 4 h after the treatments [67]. In the *A. paniculata* cell culture, MeJA at 5  $\mu$ M showed 5.25 higher accumulation of andrographolide content compared with control [97]. The root cultures of *Taverniera cuneifolia* treated with increasing concentrations of MeJA (1.0, 2.5, 5, 10, 100, and 1000  $\mu$ M) [135]. The glycyrrhizic acid content increased gradually with increase in MeJA (1–100  $\mu$ M) concentration. Approximately 2.5-fold increase in glycyrrhizic acid production was noticed in MeJA (100  $\mu$ M) treated roots, as compare to the unchallenged root culture. However, further increase in MeJA (1000  $\mu$ M) concentration resulted in decrease in glycyrrhizic acid production [135].

### 7.2. Duration of elicitor exposure

The cell suspension culture of *G. sylvestre* was treated with MeJA and SA for 24 h, 48 h, and 72 h. With the MeJA treatment, the maximum gymnemic acid production was recorded 72 h after treatment with 150  $\mu$ M ( $135.41 \pm 0.43$  mg/g DCW). The gymnemic acid content was 15.4-fold higher than the control cultures that were free of the elicitor. When the MeJA concentration exceeded 150  $\mu$ M, there was a drastic fall (36.3%) in the gymnemic acid accumulation [148]. A high concentration of 200  $\mu$ M SA was required to induce substantial quantities of gymnemic

aid ( $43.27 \pm 0.80$  mg/g DCW) in the suspensions that reached a maximum after 48 h treatment. The SA-induced response toward gymnemic acid accumulation resulted in a 4.9-fold increase in comparison to the control cultures [148]. The different biotic elicitors (*A. rhizogenes*, *Bacillus subtilis*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae*) required different duration of time (24, 48, 48, 72, and 72 h, respectively) to elicit the gymnemic acid in the cell suspension culture of *G. sylvestre* [149]. The MeJA and SA for 4 h exposure time enhanced the production of withanolide A, withanone and withaferin A content in the hairy root culture of *W. somnifera* [95]. The yields of atropine content in the *Datura metel* hairy roots were increased by nanosilver as an elicitor after 12, 24, and 48 h, but atropine accumulation in *D. metel* hairy roots was reduced by AgNO<sub>3</sub>, *Bacillus cereus*, and *Staphylococcus* after 12, 24, and 48 h [150]. In the cell culture of *Andrographis paniculata*, the MeJA induced the highest accumulation of andrographolide at 24 h compared with 48 and 72 h of treatments [97]. In the cell system of *V. vinifera*, a rapid accumulation of trans-resveratrol was recorded with MeJA treatment, starting from 2 h and reaching its maximum value at 96 h and the highest levels of viniferins recorded in cell cultures elicited with chitin (chitosan) for 144 h [91]. The MeJA produced the highest amount of bacoside A, 1.5-fold higher than the control shoots in the *B. monnieri* shoot culture after 48 h [96]. MeJA elicitation can cause an initial rapid increase in amount of various secondary metabolites from 24 to 72 h compared to controls after which a subsequent decrease can be found [151].

### 7.3. Age of culture

Age of culture plays is an important parameter in the production of bioactive compounds by elicitation. The treatment with MeJA and SA in the hairy root culture of *W. somnifera* showed highest accumulation of withanolide A, withanone, and withaferin A content after 40 days of culture [95]. In a study, 20-day-old cell cultures of *C. roseus* showed higher yields of ajmalicine on elicitation. The optimum level of ajmalicine (166 µg/g DW) was observed in 20-day-old cells elicited with extracts of *Trichoderma viride* followed by 90 and 88 µg/g DW ajmalicine in cells elicited with *A. niger* and *F. moniliforme*, respectively [127,152]. A similar type of observation was noticed from various workers Rijhwani and Shanks [153] and Ganapathi and Kargi [142]. The selenium addition at inoculum time did not significantly affect ginseng saponin accumulation. However, the addition of 0.5 mM selenium as an elicitor, after 21 days of culture, ginseng saponin content and productivity increased to about 1.31 and 1.33 times control levels, respectively [146]. The MeJA, at a concentration of 10 µM and 100 µM when introduced to cell suspension of *C. roseus* on day 6 of cell growth increased ajmalicine and serpentine production, respectively, re-elicitation showed a negative effect on both growth and alkaloid synthesis [154].

### 7.4. Nutrient composition

The composition of the medium or selection of medium also played a vital role in elicitation process. In the callus culture of *Erythroxylum coca*, the amounts of cocaine, cinnamoylcocaine, chlorogenic acid (CGA), and 4-coumaroyl quinate (CQA) were significantly affected by the

culture medium [155]. Cocaine production was nearly an order of magnitude greater on Anderson rhododendron medium (ARM) [156], Gamborg B5 (GB5) [157], and Murashige–Tucker medium (MMT) [158], but the amounts produced on MMT and GB5 were not significantly different from each other. Cinnamoylcocaine was affected in the same way. The major factor controlling tropane alkaloid (TA) accumulation was medium composition, with cocaine levels on ARM being nearly an order of magnitude greater than on the other media. Many nutrients, including cobalt, copper, molybdenum, calcium, magnesium, iron, boron, iodine, manganese, zinc, and myo-inositol, and the growth regulators and the ammonium:nitrate ratio are at equivalent levels in ARM as in one of the other media, and can therefore be excluded as factors promoting TA accumulation. However, a number of factors differed between ARM and the other media, and might be responsible for the elevated TA content. Total ion concentration is lower in ARM, and could be an important factor given the importance of salt content in controlling secondary metabolism [155]. Nitrate concentration was also lower in ARM, and there are numerous reports in the literature of an inverse relationship between nitrate availability and accumulation of secondary metabolites in many plant species, including *Arabidopsis thaliana* [159], *Hordeum vulgare* [160], and *Nicotiana tabacum* [161]. Similarly, the reduction of nitrate concentration in the culture medium of *Atropa belladonna* hairy roots increases alkaloid content [162]. In regards to CGA, the media were all significantly different from each other with the lowest production on ARM and highest on MMT. Less CQA was produced on ARM than on either of the other two media, which did not differ from each other [155]. Apart from these characteristics, the efficiency of elicitation also depends on elicitor specificity, cell line or clones of microbial elicitor used the presence of growth regulators, nutrient composition of the medium, and the environmental conditions.

## 8. Conclusion

The development of plant tissue cultures for the production of secondary metabolites has been underway for more than three decades. Although there are well-established plant tissue culture techniques, their application to large scale production is still limited to a few processes. Various stimulation and process strategies have been exercised to improve secondary metabolite production in plant tissue cultures. Elicitation has been widely applied for enhancement of secondary metabolite production in plant cell and organ cultures. The effects of various abiotic and biotic elicitors on secondary metabolite production in plant tissue cultures are dependent on the specific secondary metabolites. The exploration of the production of useful secondary metabolites through regulation of biosynthetic pathway of the various plant cell and tissue cultures of medicinal plants has been carried out by a group of plant scientists in several countries during the last decade. Although, elicitation enhances secondary metabolism in plant cells *in vitro*, but the exact mechanism is not exactly understood. There is a tremendous scope for the large-scale production of secondary metabolites in the plant tissue culture system by using the elicitors as an agent.

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## **Consequences of Water Deficit on Metabolism of Legumes**

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Additional information is available at the end of the chapter

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### **Abstract**

The aim of this chapter is (i) to define water deficit and its consequences on growth and development of higher plants; (ii) to present the interferences induced on metabolism, including gas exchange, biochemical compounds, and osmotic substances; and (iii) to explain how these alterations will affect the growth, development, and production of leguminous plants. This chapter reports that the performance in leguminous is affected by water deficiency, which can cause lower growth and development. For gas exchange, leaf relative water content, stomatal conductance, and transpiration rate suffered decrease when cultivated under water deficit. Biochemical compounds, such as soluble amino acids, soluble proteins, proline, and abscisic acid (ABA), are intensively modified after a period under water restriction. The results prove that ABA mediates actively and significantly the proline accumulation and consequent osmotic adjustment in *Vigna unguiculata* leaves that were induced to water deficit and rehydration.

**Keywords:** Abiotic stress, leguminous crops, gas exchange, osmotic adjustment, water deficiency

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### **1. Introduction**

The water supplement inadequate in soil is considered one of the limiting factors to the productive potential in several species [1–2]. Water deficit is an abiotic factor that affects the agricultural production with high frequency and intensity, influencing morphological, physiological, and biochemical aspects [3–4].

As in other crops, performance in leguminous is affected by water deficiency, which can cause lower growth and development, with progressive reduction in leaf dry matter [5], moreover, to promote the abortion of flowers during drought periods and to affect the yield significantly [6], with consequent repercussion on production parameters, such as number of grains and pods per plant.

The deficit water is characterized by water losses that exceed the absorption rate and by this way acts directly in the plant–water relations [7–8], depending on intense and exposure period, in addition to promote changes in the cell and molecular pathways [9], whereby accumulation of organic solutes with the carbohydrates and proline [10], differential gene expression of DNA [11], and quantity variation in the photosynthetic pigments, mainly chlorophylls and carotenoids [12], in which the stomata enclosed interfere in photosynthetic rates occur [13].

The osmotic adjustment is considered one of the important mechanisms developed by the plants to tolerate the water deficiency [14], which promotes the protection of the plant cell structures with membranes and chloroplasts [15], as well as avoid the cell toxicity provoked by the free radicals and maximize the water retention in cell inside [16]; besides it has the advantage of using carbohydrates as energy source under severe stress [6].

Drought is directly related to the overproduction of reactive oxygen species (ROS) [17], such as hydrogen peroxide ( $H_2O_2$ ) and superoxide ( $O_2^-$ ) [18], which are highly toxic compounds. ROS promote the oxidation of membranes and damage essential organelles such as chloroplasts [19] and mitochondria [20], which result in cell damage or death [21,22].

Ascorbate (ASC) and glutathione (GSH) have essential functions in antioxidant metabolism [23,24] because ASC is used as a substrate [25–27]. In addition, GSH produces ASC and glutathione disulfide (GSSG), which is used to regenerate GSH via glutathione reductase (GR) [28,29].

The soybean is considered a species sensitive to several abiotic stresses [30], when compared with other tropical legumes, such as *Vigna unguiculata* and *Phaseolus vulgaris* [31,32], as well as other species such as *Gossypium hirsutum* and *Sorghum bicolor* [33,34], in which the sensitivity at water deficit can be emphasized, mainly during the growth and development period, which might cause strong reduction in the yield [35]. However, *V. unguiculata* (L.) Walp. is a species tolerant to drought due to rusticity, and it presents large protein content in grain. This crop is frequently found in agricultural areas in Brazil that are under the influence of abiotic stresses. These areas present small rain index and high temperature. In addition, the soil is susceptible to salinity or to fertility loss [36].

## 2. Objectives

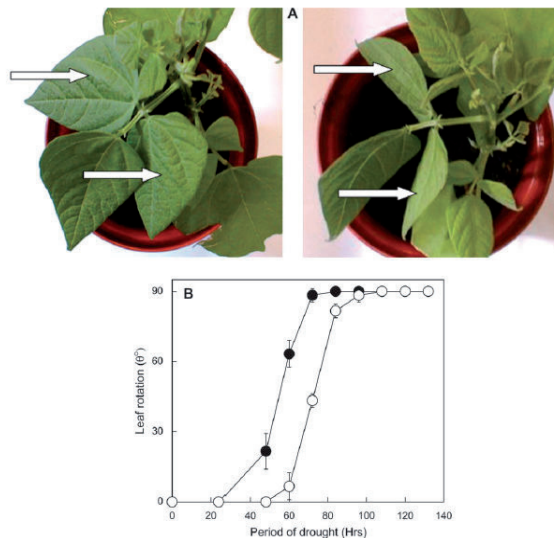
The aim of this chapter is (i) to define what is water deficit and the consequences on growth and development of higher plants; (ii) to present the interferences induced on metabolism, including gas exchange, biochemical compounds, and osmotic substances; and (iii) to explain



how these alterations will affect the growth, development, and production of leguminous plants.

### 3. Interference of water deficit on growth and development

Lizana et al. [37] while working with two varieties of *P. vulgaris* under water deficit observed paraheliotropic leaf movement, which was previously described by Pastenes et al. [38]. Leaf movements in Arroz and Orfeo subjected to drought were shown and compared. Figure 1B presents the evolution of the movement of leaves after increasing periods of drought, being determined that the variety Arroz is more sensitive than Orfeo [37].



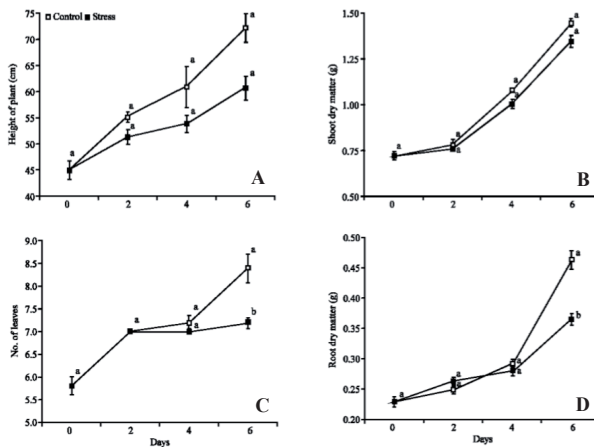
**Figure 1.** Leaf movement in different drought times. (A) Plants before (left) and after (right) drought-induced leaf movement. Leaf rotation was measured on flanking leaves (arrows) of the first mature trifoliate leaves. (B) Relationship between period of drought and leaf rotation angle (h) in Arroz (closed symbols) and Orfeo (open symbols) [37].

Lobato et al. [39] while studying morphological alterations in *Glycine max* under progressive water stress found variations in the following parameters: (A) evaluated height of plants, (B) shoot dry matter, (C) number of leaves, and (D) root dry matter.

The lower height and shoot dry matter in the plants under water deficiency occurred, probably due to the abscisic acid (ABA) action, in which case it is produced in the cells under abnormal conditions and this way inhibited the cell division and/or DNA synthesis [39].

The smaller number of leaves showed in the plants under water stress occurred with consequence a lower or void extension rate of the leaf area existent in the plant, moreover probably

increase in the ABA levels in roots, in which it will be transported from roots to shoot and act in the apical region of the plant with antagonist of the auxin and cytokinin, responsible for growth and cell division, respectively [40]; through these hormonal mechanisms, the buds remain dormant and develop not the leaf news. In the period between 0 and 2 days of water stress (Figure 2D), the weight higher of the root dry matter. According to Kerbauy [41], studies with gene-modified plants describe a decrease in the ethylene levels and increase in the ABA in plant roots under water stress, when compared with plants normally irrigated; hence, it proves the different behavior of these hormones, besides it are attributes at ABA the capacity of the remain ethylene normal levels produced in root of plants under normal conditions.



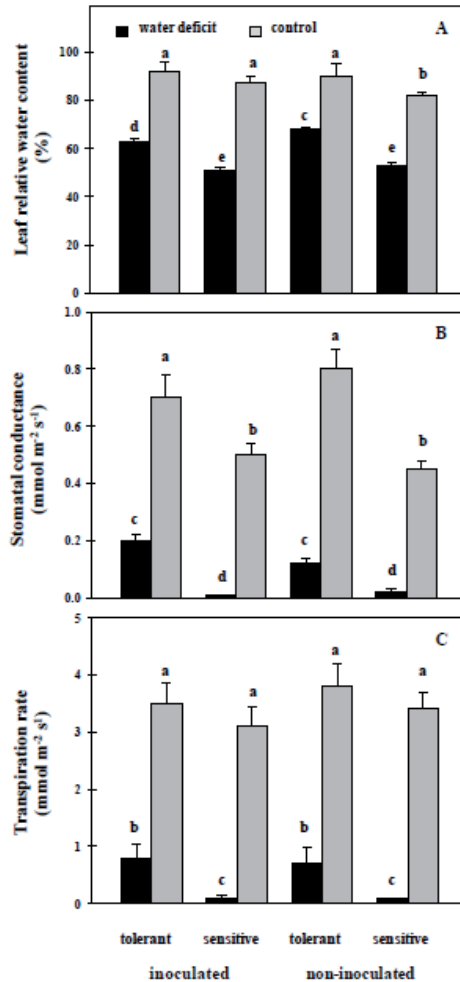
**Figure 2.** (A) Height of plants, (B) shoot dry matter, (C) number of leaves, and (D) root dry matter in plants of *Glycine max* cultivar sambaiba under 0, 2, 4, and 6 days of water stress. Averages followed by the same letter do not differ among themselves by the Tukey's test at 5% of probability, and the bars represent the mean standard error [39].

#### 4. Modifications on gas exchange

Barbosa et al. [42] evaluated the root contribution to water relations and shoot in two contrasting *V. unguiculata* cultivars and showed that water deficit promoted significant decrease in leaf relative water content (Figure 3A) in tolerant and sensitive cultivars. Inoculated plants of control treatment presented higher values of leaf relative water content, when compared with same treatments of non-inoculated plants.

The tolerant cultivar showed better performance in this parameter, when compared with that of same treatments the cultivar that is sensitive to water stress. In both tolerant and sensitive cultivars, stomatal conductance had a significant reduction in plants exposed to water deficiency (Figure 3B). Plants that were inoculated presented non-significant difference, when compared with that of non-inoculated plants.

Water restriction produced a significant decrease in transpiration rates in both cultivars (Figure 3C). The inoculation provoked non-significant changes in tolerant and sensitive plants. When the tolerant cultivar was submitted to water deficit, the values were higher than those found in the sensitive cultivar, this behavior being similar in inoculated and non-inoculated plants.



**Figure 3.** (A) LRWC, (B)  $g_s$  and (C)  $E$  in two contrasting *Vigna unguiculata* cultivars under water deficit and subjected to inoculation. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% of probability. The bars represent the mean standard error [42].

The reduction in relative water content in leaf is because of lower absorption rate of water by plant via roots and water loss occasioned by gas exchanges through stomata [43]. Similar results were reported by Maia et al. [44] when working with *Zea mays*.

Water deficit promoted a significant fall in stomatal conductance of the two cultivars, but tolerant plants presented higher values of this variable, probably by maintaining better plant water condition. This study revealed that root dry matter exercises influence on stomatal conductance in *V. unguiculata* plants submitted to 5 days of water deficiency, and this fact is based on the indirect effect produced by root on stomatal mechanisms. In other words, an insufficient root system developed during water deficiency will supply lower amount of water to shoot and, consequently, will promote reduction in stomatal conductance.

Decrease in stomatal conductance is explained by reduction in water availability in substrate, and it produces a reduction in leaf water potential, with consequent stomatal closing. The results described by Santos and Carlesso [4] reported that on conditions of water deficit, there is an increase in ABA concentration in xylem sap, promoting stomata closing.

Gholz et al. [45] reported that stomatal closing reduces the CO<sub>2</sub> influx to leaf, affecting production, transport, and utilization of photo-assimilates, and hence the yield. Results similar to those found in this study were found by Santos et al. [46], who studied five *P. vulgaris* genotypes subjected to water deficiency.

Decrease in transpiration rate of *V. unguiculata* plants can be attributed to stomatal behavior, because under water deficit, stomata are kept partially closed, contributing to change in transpiration behavior of plant [47]. Leite and Filho [48] reported that reduction of transpiration is an important mechanism of tolerance to drought. Values of transpiration demonstrated direct relation with stomatal conductance and also with leaf relative water content. Similar results were shown by Nogueira et al. [49] in a study of two *Arachis hypogaea* cultivars exposed to water deficiency.

## 5. Water deficit on nitrogen compounds

Lobato et al. [50] evaluated the effects of the progressive water deficit, as well as investigated the physiological and biochemical behavior in *G. max* cv. Sambaiba that was submitted to water restriction during the vegetative phase (Table 1). The increase in the levels of free amino acids is due to high synthesis of amino acids from protein hydrolyses, in which case the free amino acids are utilized by the plant to reduce the effects of the water deficit through organic solute accumulation, thereby increasing the water retention capacity [51].

Under water stress, the free amino acids such as proline and glycinebetaine are strongly influenced and consequently quickly accumulated [52], as well as of secondary form occur the increase of aspartate, glutamate and alanine [53]. The result on increase in the free amino acids found by Asha and Rao [54] while working with *Arachis hypogaea* under water deficit corroborates the results of this study.

Free amino acids ( $\mu\text{mol g}^{-1}\text{DM}$ )		
Days	Control	Stress
0	10.1 $\pm$ 3.5 a	10.1 $\pm$ 3.5 a
2	10.2 $\pm$ 2.8 a	41.6 $\pm$ 3.0 b
4	9.9 $\pm$ 1.9 a	41.2 $\pm$ 3.1 b
6	10.1 $\pm$ 1.5 a	49.3 $\pm$ 2.4 b

**Table 1.** Free amino acids and proline in *Glycine max* plants (cv. Sambaiba) under 0, 2, 4, and 6 days of water deficit. Averages followed by the same letter do not differ among themselves by Tukey's test at 5% of probability [50].

The reduction in the total soluble proteins showed in the plants under water stress is due to probable increase in the proteases enzyme activity (Table 2), in which case this proteolytic enzyme promotes the breakdown of the proteins and, consequently, decreases the protein amount presents in the plant under abiotic stress conditions [55]. In inadequate conditions to the plant is active the pathway of proteins breakdown, because the plant use the proteins to the synthesis of nitrogen compounds as amino acids that might auxiliary the plant osmotic adjustment [56]. Similar results on reduction in the proteins were found by Ramos et al. [57], investigating the effects of the water stress in *P. vulgaris*.

Soluble proteins ( $\text{mg g}^{-1}\text{DM}$ )		
Days	Control	Stress
0	9.74 $\pm$ 0.11 a	9.74 $\pm$ 0.11 a
2	10.05 $\pm$ 0.37 a	7.69 $\pm$ 0.09 b
4	9.87 $\pm$ 0.26 a	7.73 $\pm$ 0.19 b
6	9.71 $\pm$ 0.22 a	7.79 $\pm$ 0.21 b

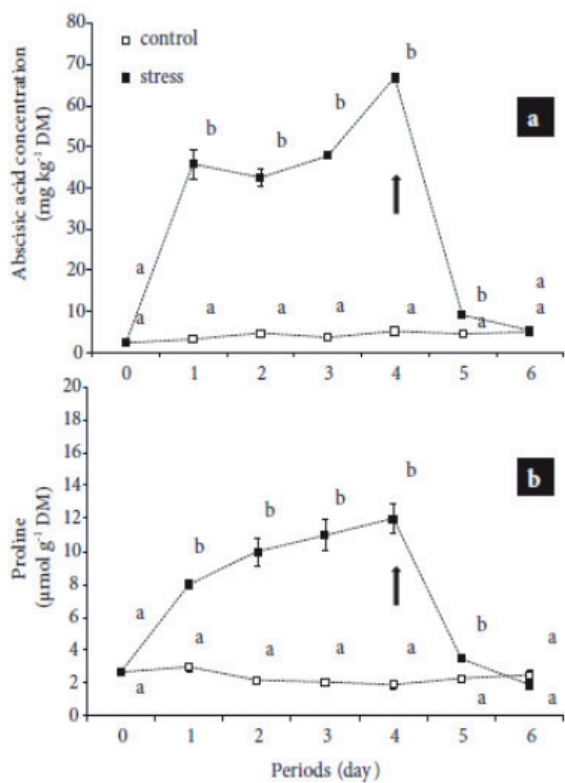
**Table 2.** Total soluble proteins in *Glycine max* plants (cv. Sambaiba) under 0, 2, 4, and 6 days of water deficit. Averages followed by the same letter do not differ among themselves by Tukey's test at 5% of probability [50].

## 6. Relation between abscisic acid and proline

The ABA hormone is synthesized in the plastids and is linked to the stomatal mechanism [58] and quickly responds to water deficiency [59]. The ABA can be produced in the roots and/or shoots, but this hormone is usually synthesized under water deficiency in the roots and translocated to leaves in order to improve stomatal control. The ABA signalization pathway depends on the  $\text{Ca}^{2+}$  influx into the cytosol [60], activating the  $\text{K}^{+}$ ,  $\text{Cl}^{-}$ , and malate<sup>-2</sup> efflux channels to external medium, through plasmatic membranes and concomitantly blocking the  $\text{K}^{+}$  entrance to cytosol. Therefore, the cytosol solute flux in the direction of the cell wall results in a decrease of turgescence pressure in the guard cells, and, consequently, the stomata are closed [61].

The progressive increase in ABA concentration in the plants of the stress treatment is related to the stomatal mechanism because this hormone, under these conditions, provokes stomatal closing [62], consequently reducing the water losses during the gas exchanges in essential physiological processes such as transpiration and photosynthesis [59].

Based in study carried out by Costa et al. [5] on impact of water deficit and rehydration on nitrogen compounds and ABA in *V. unguiculata* leaves (Figure 4), the research detected that leaf relative water content influences ABA concentration present in the leaf.

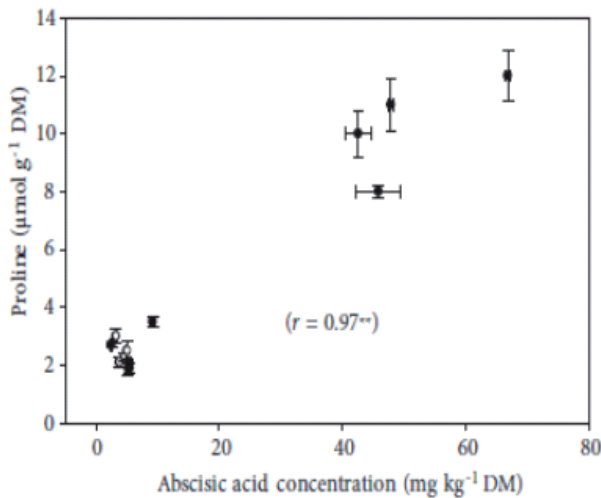


**Figure 4.** (a) Absciscic acid concentration and (b) proline in *Vigna unguiculata* plants cv. Vita 7 subjected to 4 days of water restriction and 2 days of rehydration. Means followed by the same letter are not significantly different by Tukey's test at 5% of probability. The bars represent the mean standard error, and the arrow indicates the rehydration point [5].

Therefore, the relative water content acts as a signal, and the ABA works during the signal transduction due to the easy and fast movement of this compound into plant tissue, and as a response, the stomatal closing occurs in *V. unguiculata* plants subjected to water deficit.

The fast decrease in the ABA concentration after rehydration indicates the efficiency of the signalization pathway, transduction, and consequent response of this compound. The results reported in this study on ABA are corroborated by Hsu et al. [63] evaluating the consequences of water stress in *Oryza sativa* L. and the effects of heavy metal stress in *Cicer arietinum* L.[64].

The results obtained by Costa et al. [5] prove that ABA mediates actively and significantly the proline accumulation and consequent osmotic adjustment in *V. unguiculata* leaves induced to water deficit and rehydration (Figure 5). A recent study indicated that *V. unguiculata* plants considered resistant to water deficit presented proline accumulation [65] and, consequently, are more adapted to environments with low water supplement, when compared to sensitive plants. The rehydration reduced the proline levels, suggesting that this nitrogen compound participates actively in the osmotic adjustment in this species. The proline accumulation during water deficit presented in this study is similar with results reported by Sarker et al. [66], investigating *Triticum aestivum*, and Smita and Nayyar [67], evaluating *C. arietinum*.



**Figure 5.** Relationship between abscisic acid concentration and proline in *Vigna unguiculata* plants cv. Vita 7 subjected to 4 days of water restriction and 2 days of rehydration. The bars represent the mean standard error, and the asterisks (\*\*) indicate the significance at 0.01 probability level [5].

## 7. Final considerations

This chapter reports that the performance in leguminous is affected by water deficiency, which can cause lower growth and development, with progressive reduction in leaf dry matter, moreover to promote abortion of flowers during drought periods, and to affect the yield

significantly, with consequent repercussion on production parameters, such as number of grains and pods per plant. In relation to morphological parameters, negative alterations related to height of plants, shoot dry matter, number of leaves, root dry matter, and paraheliotropic leaf movement were described. For gas exchange, leaf relative water content, stomatal conductance, and transpiration rate suffered decrease when cultivated under water deficit. Biochemical compounds, such as soluble amino acids, soluble proteins, proline, and ABA, are intensively modified after a period under water restriction. The results prove that ABA mediates actively and significantly the proline accumulation and consequent osmotic adjustment in *V. unguiculata* leaves that were induced to water deficit and rehydration.

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## Glycation of Plant Proteins under Environmental Stress — Methodological Approaches, Potential Mechanisms and Biological Role

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Additional information is available at the end of the chapter

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### Abstract

Environmental stress is one of the major factors reducing crop productivity. Due to the oncoming climate changes, the effects of drought and high light on plants play an increasing role in modern agriculture. These changes are accompanied with a progressing contamination of soils with heavy metals. Independent of their nature, environmental alterations result in development of oxidative stress, i.e. increase of reactive oxygen species (ROS) contents, and metabolic adjustment, i.e. accumulation of soluble primary metabolites (amino acids and sugars). However, a simultaneous increase of ROS and sugar concentrations ultimately results in protein glycation, i.e. non-enzymatic interaction of reducing sugars or their degradation products ( $\alpha$ -dicarbonyls) with proteins. The eventually resulting advanced glycation end-products (AGEs) are known to be toxic and pro-inflammatory in mammals. Recently, their presence was unambiguously demonstrated *in vivo* in stressed *Arabidopsis thaliana* plants. Currently, information on protein targets, modification sites therein, mediators and mechanisms of plant glycation are being intensively studied. In this chapter, we comprehensively review the methodological approaches for plant glycation research and discuss potential mechanisms of AGE formation under stress conditions. On the basis of these patterns and additional *in vitro* experiments, the pathways and mechanisms of plant glycation can be proposed.

**Keywords:** Advanced glycation end-products (AGEs), Ageing, Environmental stress, Glycation, Proteomics

## 1. Introduction

### 1.1. Environmental stress, ROS and protein glycation

#### 1.1.1. Environmental stress and ROS formation

Environmental stress is one of the major factors reducing the productivity of crop plants all over the world [1]. Drought, high light, salinity and increased heavy metal soil contents, as well as extreme temperature, represent its important manifestations [2]. On the physiological level, extreme environmental conditions ultimately results in decrease in the CO<sub>2</sub> assimilation rate and in growth inhibition [3]. Simultaneous accumulation of reduced equivalents results in an overload of the chloroplast and mitochondrial electron transport chains and enhanced production of the reactive oxygen species (ROS), i.e. singlet oxygen (<sup>1</sup>O<sub>2</sub>), superoxide radical anion (O<sub>2</sub><sup>•-</sup>), peroxide ion (O<sub>2</sub><sup>2-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), various hydroperoxides and hydroxide radical (OH<sup>•</sup>) [4]. When ROS production overwhelms their detoxication, oxidative stress develops [5].

Thus, transfer of electrons to molecular oxygen (O<sub>2</sub>) from ubisemiquinone in mitochondria and thylakoid membrane-bound primary electron acceptor of photosystem I (PSI) in chloroplasts yields O<sub>2</sub><sup>•-</sup> (and, when further reduction occurs, O<sub>2</sub><sup>2-</sup>), further converted to H<sub>2</sub>O<sub>2</sub> by superoxide dismutase (SOD) activity (predominantly Mn- and CuZn-SOD in mitochondria and chloroplasts, respectively) [6,7]. The radical oxygen species can abstract protons from (bis-)allylic methylenes of polyunsaturated fatty acids (PUFAs) [8]. The subsequent capture of O<sub>2</sub> molecule by the resulting carbon-centered radical yields a peroxyl radical, that is able to initiate a chain reaction of lipid peroxidation [9]. The PUFAs can be directly attacked by the protonated form of O<sub>2</sub><sup>•-</sup> (HO<sub>2</sub><sup>•</sup>) [10], thus the content of lipid hydroperoxides is one of the most reliable markers of oxidative stress.

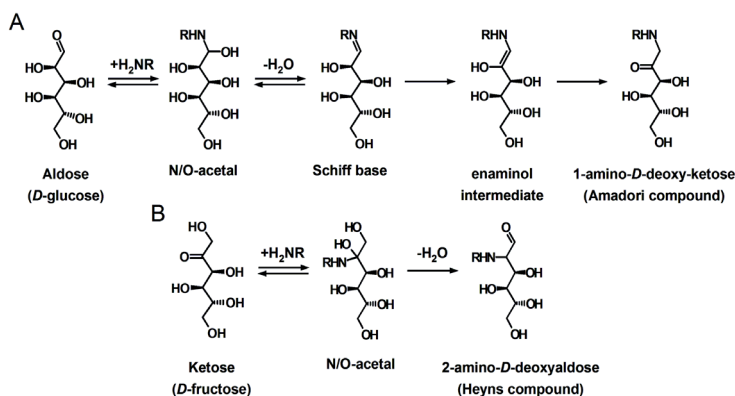
The hydroperoxides can be easily involved in the Fenton reaction, i.e. transition metal ion-mediated reduction, yielding OH<sup>•</sup>, i.e. one of the most short-living and toxic ROS, directly and irreversibly modifying lipids, proteins and nucleic acids [11]. The metal ions oxidized during Fenton reaction are reduced *in vivo* by cellular antioxidants or O<sub>2</sub><sup>•-</sup> (Haber-Weiss reaction), that considerably increases the production of OH<sup>•</sup> [12]. As environmental stress is accompanied with a strong upregulation of mono- and oligosaccharides in all plant tissues [13], metal-catalyzed oxidation of sugars (so-called monosaccharide autoxidation) [14] also might be enhanced under the conditions of oxidative stress. The resulting products – hydroxycarbonyls and α-oxocarbonyls – are the potent protein modification agents and can induce essential changes in their structure and function [15]. These reactions, termed protein glycation, i.e. modification of proteins by carbonyl compounds (carbohydrates and α-oxocarbonyls), is believed to be an important factor in stress-related protein damage [16].

#### 1.1.2. Protein glycation

In the first step of this process (usually termed “early glycation”), reducing sugars, aldoses and ketoses reversibly interact with amino groups resulting in the very labile N/O-acetal



intermediates: aldoamines and ketoamines, respectively (Figure 1). These compounds easily condense yielding aldimines and ketoimines (Schiff bases), which undergo Amadori [17] and Heyns [18] rearrangements, variants of the acyloin shift. Amadori rearrangement involves proton transfer from C1 to C2 via the enol/enamine intermediate yielding *N*-substituted 1-amino-deoxy-ketoses, the Amadori products (Figure 1A). Similarly, in the case of ketoamines, in course of Heyns rearrangement [18], a proton migrates from C2 to C1 forming 2-amino-deoxyaldosyl adducts, often referred to as Heyns products (Figure 1B) [19]. Both Amadori and Heyns products are termed as “early glycation products”, the first relatively stable intermediates of glycation [20].

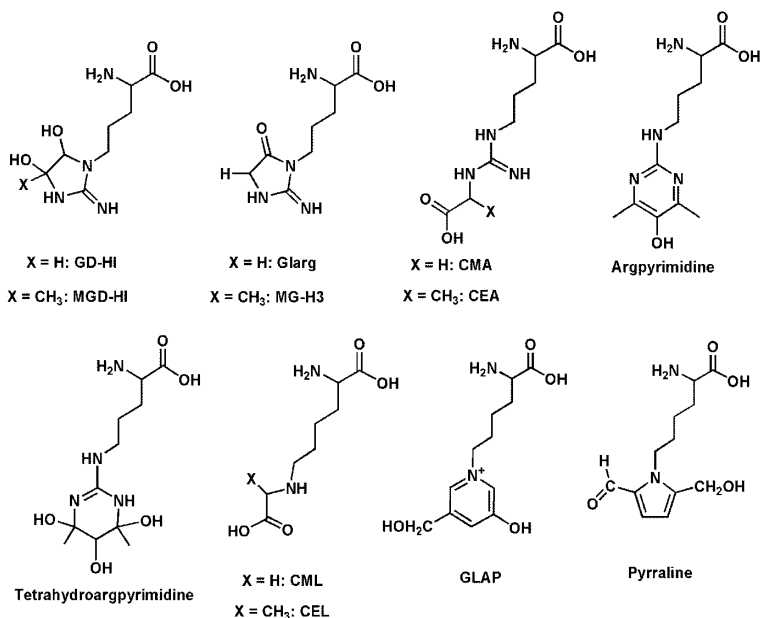


**Figure 1.** Formation of N/O-acetal and Schiff intermediates with subsequent Amadori rearrangement (A), and ketoamine intermediates with subsequent Heyns rearrangement (B, not all intermediates shown).

These early glycation products, as well as free sugars, readily autoxidize (by the mechanisms similar to those described for free sugars) with formation of highly reactive  $\alpha$ -dicarbonyl compounds (presumably glyoxal, methylglyoxal and various osones) – potent reactive intermediates of advanced glycation [21]. Depending on the structure of the carbohydrate moiety involved in this degradation, i.e. free sugars, or protein-bound early glycation products, two principle advanced glycation pathways, namely “oxidative glycosylation” and “glycoxidation”, respectively, are distinguished [14,22,23]. The interaction of  $\alpha$ -dicarbonyls with lysyl amino and arginyl guanidino side chain groups results in formation of so-called advanced glycation end-products (AGEs) – protein Maillard reaction compounds accumulating during thermal processing of food (Figure 2) [24], but also endogenously, e.g. under the conditions of persisting hyperglycemia.

### 1.1.3. Advanced Glycation End-products (AGEs)

AGEs represent a highly heterogenic group of compounds, varying greatly in their stability. Thus, the term “advanced glycation end-products” is, to high extent, conventional: some AGEs



**Figure 2.** AGEs detected *in vivo*: GD-HI, glyoxal-derived dihydroxyimide; MGD-HI, methylglyoxal-derived dihydroxyimide; MG-H, methylglyoxal-derived hydroimidazolone; Glarg, glyoxal-derived hydroimidazolone; CML, N<sup>ε</sup>-carboxymethyllysine; CEL, N<sup>ε</sup>-carboxyethyllysine; GLAP, glyceraldehyde-derived pyridinium compound.

are still reactive and can be easily involved in further reactions [25]. In the past few decades, several AGEs were comprehensively characterized (Figure 2). Among lysine-derived modifications, N<sup>ε</sup>-(carboxymethyl)lysine (CML) [26], N<sup>ε</sup>-(carboxyethyl)lysine (CEL) [27], ε-(2-formyl-5-hydroxymethyl-pyrrolyl)-L-norleucine (pyrraline) [28] and glyceraldehyde-derived pyridinium compound (GLAP) [29] are the best-characterized. Not less attention was paid to the modifications of arginine. Thus, Schwarzenbolz and coworkers reported 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine (Glarg) as a product of the reaction of arginine with glyoxal [30] and yielding N<sup>ε</sup>-carboxyethylarginine (CMA) upon hydrolysis at 37°C [31]. Methylglyoxal was shown to form isomeric methylglyoxal-derived hydroimidazolones (MG-Hs) with N<sup>ε</sup>-(5-methyl-4-oxo-5-hydroimidazolinone-2-yl)-L-ornithine (MG-H1) as the major isomer [32]. Hydrolysis of 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolone-1-yl)pentanoic acid (MG-H3) yields carboxyethyl-L-arginine (CEA) [33]. Sequential modification of arginine with two methylglyoxal molecules results in N<sup>ε</sup>-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine (argpyrimidine, Argpyr) [34] and N<sup>ε</sup>-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine (tetra-hydroargpyrimidine, TH-Argpyr) [35].

Upon their absorption in human intestine, AGEs interact with endothelial and macrophage pattern recognition receptors for AGEs (e.g. RAGEs) and trigger NF-κB-mediated expression

of pro-inflammatory species (e.g. adhesion molecules, including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1) [36] and foster the development of inflammatory diseases – e.g. atherosclerosis and type 2 diabetes mellitus [37]. AGEs of different chemical structure and origin, most often CML, pentosidine and hydroimidazolones, are known to be the ligands of RAGEs and to trigger inflammatory response [38].

Surprisingly, other reports showed mammalian serum and urinary concentrations of AGEs to be independent from dietary intake of thermally processed foods [39]. Moreover, the levels of CML and fluorescent AGEs in the plasma of vegetarian individuals were higher in comparison to those in the omnivorous individuals [40], even though the vegetarian diet had lower contents of lysine- and arginine-containing proteins. Remarkably, this effect was stronger in plasma of long-term vegetarians [41]. These facts indicate a high relevance of protein glycation (both early and advanced) in plants. Obviously, this explains the presence of multiple efficient anti-glycative enzymatic systems, like glyoxalase I and II [42], ribulosamine/erythrulosamine 3-kinase [43], acylamino acid-releasing enzyme [44].

Recently, Bechtold and co-workers reported an increase in the total contents of individual AGE classes upon the application of experimental environmental stress [16]. Thus, it is obvious that environmental changes are accompanied with enhanced generation of AGEs in plant tissues. In other words, due to the continuously altering growth conditions, AGEs might accumulate in plants during their life span, causing stress-related changes of the plant proteome and its physiological state. Important to note, that due to the dramatically different metabolic background (i.e. other patterns of carbohydrates, as well as high contents of potential antioxidants and carbonyl traps), pathways of glycation in plants may differ from those described in mammals. However, no information about the proteins and biochemical pathways affected by such glycation reactions (i.e. its structural and functional patterns) was available until very recently. The most recent studies from our labs on the protein glycation patterns of model plants in the absence and presence of environmental stresses, as well as the impact of protein glycation in plant ageing, are added to this chapter.

## 2. Methodological approaches for the study of protein glycation in plants

### 2.1. Proteomics in plant glycation research

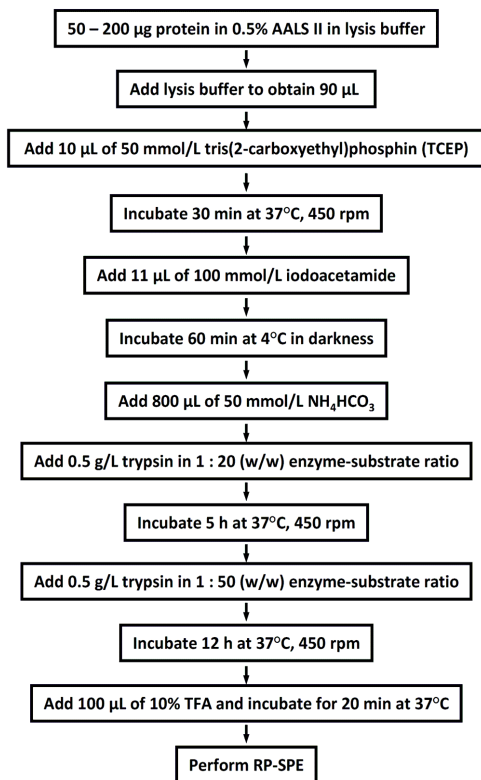
Recently, using liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based approach, Bechtold and co-workers estimated the total levels of individual AGE classes in *Arabidopsis thaliana* leaf tissues [16]. Their methodology relied on the tandem mass spectrometric (MS/MS) analysis of individual amino acids in tissue hydrolyzates by multiple reaction monitoring (MRM) [45]. Though this method provides high sensitivity and specificity, this strategy could not provide information about the identity and modification patterns of individual glycated proteins. However, this question can be addressed by the methods of LC-MS-based proteomics – the technique based on the analysis of protein enzymatic hydrolyzates

[46]. Indeed, in its classical implementation, unbiased LC-MS approach combines high separation efficiency of the high-performance or ultra-high-performance liquid chromatography (HPLC or UPLC, respectively) and outstanding resolution and mass accuracy of high-resolution mass spectrometry – Orbitrap- and quadrupole-time-of-flight (QqTOF)-MS [47,48]. It dramatically increases the analytical resolution (i.e. the number of analytes, annotated in one experiment) of the whole method. Moreover, due to the superior separation, low-abundant post-translationally modified peptides are less amenable to the ion suppression effects, accompanying electrospray ionization (ESI) [49]. Obviously, this analytical strategy seems to be the optimal for the study of such heterogenic and low-abundant set of modifications as AGEs.

## 2.2. Protein isolation and proteolysis

Depending on the target protein fraction (soluble or total), aqueous (aq.), extraction can be performed in the absence and presence of phenol, respectively [50]. In the first case, however, even the extracts of green parts contain high amounts of soluble metabolites that might inhibit the activity of proteases used for digestion. Such incompleteness of proteolysis can be observed as well, when proteins are extracted from plant parts rich in anti-nutritive (i.e. protease inhibiting or denaturing) phenolics like insoluble condensed tannins in seeds [51]. Therefore, the extracts can be purified by gel filtration chromatography and/or ultrafiltration using Centricon or Vivaspin centrifugation devices [52] prior to the determination of protein concentration. Alternatively, the proteins can be isolated by phenol extraction. In this case, phenolics contaminants can be removed by addition of 1–5% of soluble or insoluble polyvinylpyrrolidone [50]. While purified aqueous extracts can be easily digested by proteases in the presence of only deoxycholate as a denaturizing agent [53], the dried proteins isolated with phenol (containing also the fraction of hydrophobic membrane proteins) can be reconstituted only in the presence of both chaotropic compounds (urea, thiourea) and strong detergents. Conventional detergents, such as sodium dodecyl sulfate (SDS) or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), co-elute with proteolytic peptides and disturb ESI. This can be avoided by application of the detergents which do not impact protease activity and can be easily destroyed upon the digest. For example, *RapiGest*<sup>TM</sup> SF Surfactant (Waters Corporation, Milford, US) or Progenta Protea Biosciences, Inc., Morgantown, US) can be applied [54]. The detergents can be dissolved in the lysis buffer (7 mol/L urea and 2 mol/L in 50 mmol/L Tris-HCl, pH 7.5) and destroyed directly after proteolysis at pH 2 for 20–45 min (Figure 3).

The completeness of the digestion can be controlled by the SDS-polyacrylamide gel electrophoresis (PAGE). Performing SDS-PAGE additionally prior to proteolysis provides the opportunity to validate the Bradford assay results by relative quantification of total lane densities and, if necessary, normalize the results of the LC-MS-based label-free quantification. It is important to stress that the use of chaotropic agents, acid-labile detergents and strong acids for their cleavage ultimately require RP-based solid phase extraction (SPE) after completion of the digest and verification of its completeness by SDS-PAGE.



**Figure 3.** Protocol for tryptic digestion of the total plant protein obtained by phenol extraction. The procedure employed tryptic digestion in the presence of an acid-labile detergent ALLS II, required to ensure efficient solubilisation of membrane proteins.

### 2.3. LC-MS analysis of glycated peptide mixtures

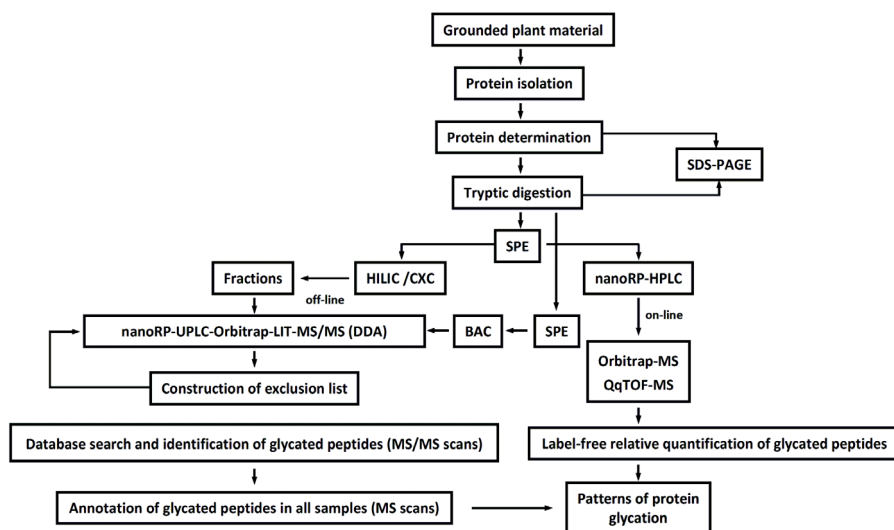
The proteolytic digests are, typically, complex mixtures. Therefore, for successful detection and identification of their components (i.e. peptides), adequate chromatographic and mass separation techniques need to be applied. The selection of a strategy for LC-MS analysis (in terms of throughput, sensitivity, separation efficiency and reproducibility) depends on the aims of particular research. In the absolute majority of cases, LC-MS analyses rely on so-called data-dependent acquisition experiments (DDA) [55]. These complex experiments comprise survey MS scan (typically performed in Orbitrap- or TOF mass analyzer), and multiple dependent ones – MS/MS, relying on linear ion trap (LIT) or QqTOF analysis [56,57]. Modern instruments provide a possibility for introduction of the second dependent scan. Thus, hybrid LTQ-Orbitrap instruments support multi-step activation (MSA) experiments, comprising an additional MS/MS scan with a low-energy collision-induced dissociation (CID) [58]. The

modification-specific neutral losses, appearing under these conditions, trigger a high-energy dependent MS/MS scan providing rich structural information. In the past decade, these experiments were successfully applied to glycosylated tryptic digests using Amadori-specific losses of two and three waters, as well as additional formaldehyde molecule [59].

In the most straightforward way, DDA experiments are performed without additional analytical procedures preceding a RP-HPLC separation – so-called shotgun proteomics. However, as well as other PTMs, glycosylative and glycosylative modifications are low abundant. Hence, their ionization might be suppressed by highly abundant species. To avoid this, early glycosylated tryptic peptides can be selectively enriched by boronic acid affinity chromatography (BAC) before LC-MS/MS analysis [60]. However, such enrichment is not possible for the whole fraction of advanced glycosylated peptides due to their structural heterogeneity. Therefore, these low-abundant species need to be directly detected in conventional data-dependent acquisition (DDA) experiments. As the DDA algorithm relies on the MS/MS analysis of the most intense signals in each time segment, this type of experiments suffers from so-called undersampling, i.e. missed fragmentation of low-abundant quasi-molecular ions [61]. Because of this reason, shotgun proteomics is not a desired strategy for the analysis of PTMs.

Thus, the number of co-eluting peptides in DDA analyses needs to be reduced to increase the coverage of the AGE-modified proteome. This can be addressed by three approaches: (i) introduction of enrichment or pre-fractionation step, (ii) gas phase fractionation (GPF) on the MS level and (iii) use of retention time-based exclusion lists. In all cases, for reliable identification of AGE-containing peptides, multiple DDA experiments are required. A special attention needs to be paid on the number of dependent MS/MS scans and the total duration of cycle. Thus, the number of MS/MS scans should not be too high, as the quasi-molecular ions of AGE peptides have relatively low intensities and require, therefore, longer fill times in LIT and accumulation time TOF. The cycle times typically need to be shorter, than in conventional shotgun DDA experiments, as the peak widths of low abundant peptides are smaller.

The generalized analytical strategy might comprise both qualitative and quantitative approaches, i.e. identification of glycosylated peptides in DDA experiments with their subsequent label-free quantification in additional full-scan MS experiments (Figure 4). For identification of glycosylation sites, early glycosylated peptides can be selectively enriched [60], while the analysis of AGE-containing species might rely on two-dimensional liquid chromatography (LC × LC) [62]. BAC is a well-established analytical tool to enrich Amadori and Heyns products from mammalian tissues (predominantly plasma) [56]. However, for application to the study of plant glycosylation, this method requires some optimization. Thus, the protein extract must be effectively washed prior to digestion (e.g. by ultrafiltration) to remove the co-extracted carbohydrate-related metabolites (mono-, oligosaccharides, sugar esters and glycosides of (poly)phenolics). Due to their *cis*-diol groups, these metabolites saturate the binding sites of the affinity column and reduce the peptide enrichment efficiency. Moreover, phenylpropanoids, as well as their sugar esters and glycosides, are well-retained on reversed phase [63] and might co-elute with peptides in course of RP-HPLC separation, leading to essential ion suppression during the ESI process and shift of peptide signals in the lower part on the instrument dynamic range. Thus, the analysis of early glycosylation proteins in total extracts that can not be efficiently ultrafiltered is a challenging task.

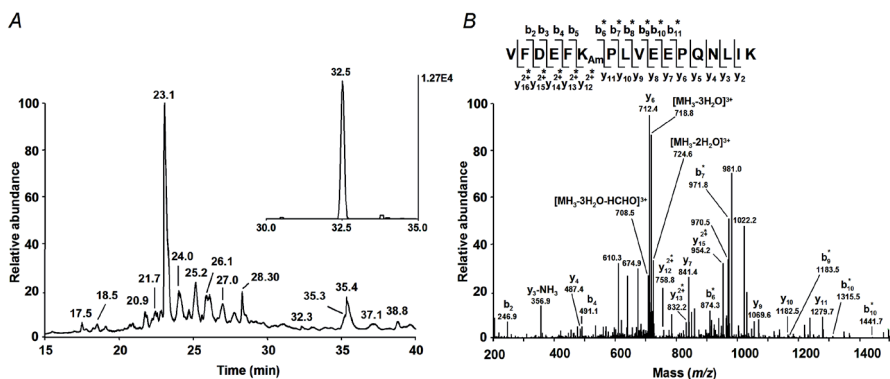


**Figure 4.** Experimental workflow for the analysis of glycosylated proteome. The analytical strategy comprises separation and MS/MS-based identification of modified peptides, as well as label-free quantification of corresponding glycosylated sites.

Pre-fractionation is usually introduced as an (relative to the RP) orthogonal separation procedure – most often cation exchange or hydrophilic interaction chromatography (HILIC) [62,64]. This approach essentially decreases sample complexity, facilitating, thereby, the fragmentation of low-abundant species. Similarly, fractionation can be applied on the MS level. Thus, simultaneously formed quasi-molecular ions (i.e. originating from the co-eluting species) can be fractionated by their  $m/z$  values in the quadrupole filter preceding LIT (GPF approach). In this case, however, repeated injections are necessary to cover the whole mass range that increases the overall analysis duration. For the most of applications, three GPF segments ( $m/z$  400–600, 600–800 and 800–1500) are sufficient to significantly improve protein identification rates [57,64]. Additionally, based on the results of one DDA experiment, all unmodified peptides can be excluded from fragmentation in following ones on software level by generation of a corresponding “exclusion list” in the instrumental method [57]. However, the combination of these approaches is required for in-depth proteome analysis. The peptides can be identified by database search using SEQUEST or Mascot search engines.

Early glycosylated tryptic peptides can be annotated in high-resolution Orbitrap-MS experiments by the  $m/z$  value and charge of the corresponding quasi-molecular ion, and retention time in corresponding extracted ion chromatograms (XICs) as shown in Figure 5A [57]. Unambiguous identification of these peptides relies on characteristic series of b- and y-ions with consideration of the losses of three water molecules (pyrylium ions) and additional loss of formaldehyde (furylium ions) [59] (Figure 5A). Identification and quantification of AGE-modified peptides are based on the same principle [65]. The only difference in comparison to the early glycation

species is the absence of the neutral losses accompanying parent and fragment ions. Additionally, the glycation state of peptides can be confirmed by characteristic fragments in the low  $m/z$  range [61,66]. This information can be further used for label-free quantification of individual AGE-modified peptides and, hence, specific glycation sites. The quantification typically relies on the integration of the annotated signals and qualitative comparisons of obtained peak areas. Thereby, the peak areas can be normalized to the signals of unmodified peptides in the same sample or in quality controls (QC). The peak integration can be performed by means of the vendor software packages – Xcalibur Quan Browser or LCQuan.



**Figure 5.** Annotation of Amadori peptides by nanoUPLC-ESI-LTQ-Orbitrap-MS/MS DDA experiments. Reversed phase ESI-Orbitrap-MS total ion chromatogram and the segment of the extracted ion chromatogram (insert) for  $m/z$   $736.39 \pm 0.02$  in the  $t_R$  range of 30–35 min (A) and an ESI-LTQ-MS/MS spectrum at  $m/z$  736.4 corresponding to the  $[M+3H]^+$  ion of the peptide VFDEFFK<sub>Am</sub>PLVEEPQNLIK (B) acquired at 32.5 min of the same DDA experiment. Pyrylium b and y fragment ions are marked with asterisk [61].

The physiological role of glycation can be assessed by the system biology software tools. Thus, for the grouping of AGE-modified proteins by their functions, the mapping software MapMan (Max-Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany, <http://mapman.gabipd.org>) can be used. The functional annotation of proteins might give insight in biological effects of AGEs in plants and provide the material for future biological studies. Thus, the proteomic data can be complemented by the result of transcriptional analysis and determination of enzymatic activities. Afterwards, the functional role of glycation in respect of particular proteins can be confirmed by the experiments with corresponding mutants.

### 3. *In vivo* glycation of plant proteins

#### 3.1. Plant protein glycation patterns

The possibility of plant protein glycation is considered since the beginning of the past decade, when Yamauchi and co-workers proposed the formation of AGEs as one of the possible



mechanisms underlying inactivation of ribulose biphosphate carboxylase/oxidase (RuBisCO) by high light [67]. Thereby, they proposed ascorbic acid as a possible precursor of AGEs. Indeed, this highly abundant compound in plant tissues can easily autooxidize and is recognized, therefore, as a potent glycation agent [68]. However, besides ascorbic acid, photosynthetically active leaf tissues contain high amounts of highly reactive pentoses, tetroses and trioses, as well as their phosphorylated forms, that might be even more reactive [69]. Probably, these sugars could be an important factor of light-dependent glycation. Recently, Bechtold and co-workers provided *in vivo* confirmation of this assumption: the authors found that tissue fructosyl lysine contents (determined by LC-MS/MS in exhaustive enzymatic protein hydrolyzates) are approximately four-fold higher in the day time in comparison to the dark period. It was not, however, the case for AGEs – just minor changes in their contents were observed during the day.

Implementation of the proteomic approach resulted in identification of proteins involved in glycation and exact modification sites therein. This strategy allowed identification of several hundreds of polypeptides containing early glycation moieties. Interestingly, the number of modification sites was higher in *Arabidopsis thaliana* in comparison to its close relative *Brassica napus*. Thus, glycation patterns might vary between species, although in both cases they are dominated by triose- and tetrose-derived products, accompanied with less abundant groups of pentose-modified sites, while hexose-derived modifications (typically the most representative in mammals) were less abundant.

Surprisingly, in plant proteins, the numbers of AGE-modified residues are essentially higher in comparison to the early glycated sites: approximately three- and seven-fold differences were observed for *A. thaliana* and *B. napus* proteome, respectively. It is the principle difference from glycation in mammals: though thousands of early glycated proteins were identified in human plasma and red blood cell membranes [64,70], only several dozens were proved to be AGE-modified [61,66]. Interestingly, the AGE modification sites in plant proteins are not accompanied with their early glycated counterparts, and are mostly originating from glyoxal and methylglyoxal. This situation differs drastically from the observations done with mammals. Indeed, several confirmed AGE sites in blood proteins (at least those representing the major plasma polypeptide human serum albumin, HSA) resembled the early glycated residues, indicating glycooxidation as an important pathway of AGE formation *in vivo*. The absence of such glycation sites in plant proteome clearly indicates the early glycation products as unlikely precursors of AGEs in plants. In this context, oxidative glycosylation rather than glycooxidation might be the predominant AGE formation pathway in plants. Remarkably, the number of early glycated lysyl residues was not only absolutely but also relatively (in comparison to the number of AGE-modified sites) lower in *B. napus*, than in *A. thaliana*. Most probably, it indicates higher activities of deglycation enzymes in the former plant.

It was shown in mammals that the proteins controlling gene expression (e.g. transcription factors or the molecules involved in protein metabolism) can be the targets of glycation [71]. The same was demonstrated for plants. This might indicate the involvement of AGE formation in the regulation of gene expression on the levels of transcription and protein biosynthesis. This can be explained by the role of protein degradation in AGE metabolism and high

representation of arginyl residues in the transcription factors that makes these molecules highly amenable to interaction with  $\alpha$ -dicarbonyls [71].

3.2. Protein glycation and environmental stress

3.2.1. Experimental models for the study of plant glycation

The main environmental stresses the plant can encounter in its habitat are high irradiation, contamination with heavy metals or metalloids and drought. It is necessary to take into account that drought is a general manifestation accompanying water deficit and is characteristic for the response to some other environmental factors, like high salt contents in soil or extremely low or high temperatures [72]. Obviously, for the study of any stress-specific response, selection of an appropriate model is of the principle importance. In this context, the researcher needs to be able to define all stress parameters by the selected experimental setup. This can be relatively easily achieved for a high light stress by using a phytotron equipped with the lamps providing required light intensity and complete climate control. In this case, a soil model can be applied (Figure 6) [73]. However, this approach does not provide the conditions equal for all plants, when a heavy metal stress is applied. That is why, growth of plants in aqueous (aq.) culture with a subsequent addition of a heavy metal salt to a growth medium seems to be a more adequate solution [74] (Figure 6).

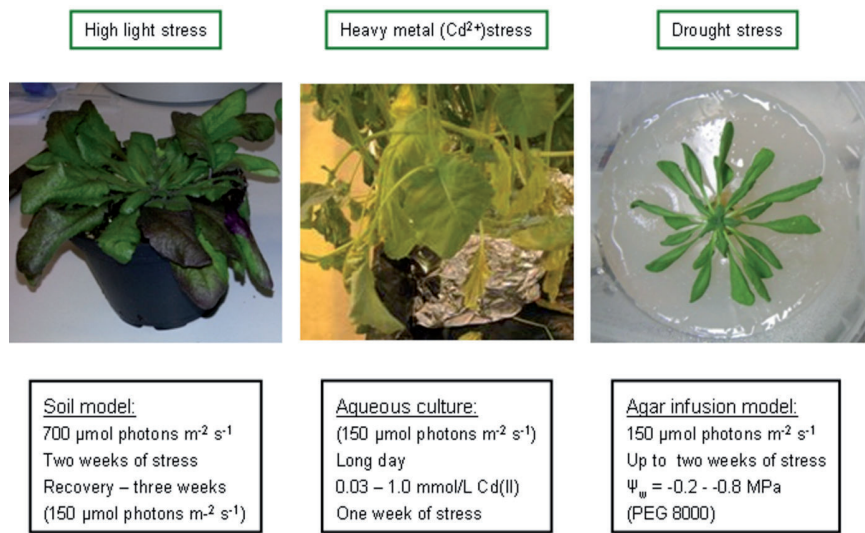


Figure 6. Experimental models of environmental stress.

As far as the drought stress is concerned, the water deficit can be modeled both in soil and in aqueous systems. According to Boyer [75], this water deficit can be expressed as a decrease of

water potential ( $\Psi_w$ ), i.e., as a difference of water potentials in a solution and pure water divided by partial molar volume of water. However, the soil-based approaches do not allow experimental definition of  $\Psi_w$ , as in this case it depends from the water absorption by plant. In aqueous culture, osmotically active substances, like commonly used sorbitol or polyethylene glycol (PEG), can affect the function of root epidermis [72]. Because of this, agar-based PEG infusion model, introduced in the past decade by van der Weele and co-workers [76], and additionally optimized to mature plants (Figure 6) seems to be more suited for this purpose. The confirmation of the stress development might rely on the determination of native leaf fluorescence [77], hydrogen peroxide contents [16] and some further parameters.

### 3.2.2. Stress-related glycation of plant proteins

Light is one of the most essential factors determining plant growth, development and survival [78]. However, a continuous exposure of plants to the high doses of sun irradiation might exceed the capacity of the mesophyll photosynthetic apparatus and trigger development of the oxidative stress [7]. In the context of the protein Maillard chemistry, discussed above, it is logical to assume that enhanced ROS and monosaccharide production would lead to the increase of AGE formation in plant green tissues. As was proposed earlier, such modifications of the RuBisCO polypeptide chains might impact inactivation of the enzyme with high light [67]. Similar mechanisms might accompany the development of drought and metal stress. Moreover, the tissue metal-scavenging mechanisms include activation of the enzyme phytochelatin synthase, requiring GSH as a substrate [79]. Hence, glyoxalase system, critical for detoxication of methylglyoxal (MGO) and utilizing GSH as a substrate as well [80], can not perform efficient scavenging of this dicarbonyl under stress conditions, which might stimulate enhanced glycation by MGO in the proteins of metal-treated plants.

The effect of stress on the formation of AGEs differs on the qualitative and quantitative levels. Thus, in most cases, relatively low number of stress-specific glycation sites can be detected in the proteins of stressed plants, and such sites are representing mostly the molecules involved in transcription and protein degradation (i.e. those known to be upregulated under stress conditions) [81]. This could be explained by the activation of some unknown enzymatic systems eliminating either AGE precursors, or AGE-modified proteins, or their early glycated precursors, i.e. Amadori and Heyns compounds. Indeed, activation of the glyoxalase system (comprising enzymes glyoxalase I and II) is well documented during environmental stress [82]. Additionally, although in mammals advanced glycation decreases the rates of proteolysis, in plants it can be not the case, that was confirmed by *in vitro* experiments [67]. Thus, acylamino acid-releasing enzyme might impact in plant protein degradation pathways [44]. However, the pathways, involved in enhanced degradation of glycated proteins in plants still need to be studied. Finally, Amadori and Heyns products can be degraded via phosphorylation pathway [43]. Besides, the possibility of the further reactions of AGEs to form new unknown products also needs to be considered [83].

Compared to the qualitative alterations, the quantitative stress-related changes in glycosylated proteome are much more pronounced. Thus, several AGE classes were at least two-fold increased even after short application of light stress [16]. This tendency could be followed on the level of individual glycation sites. However, changes in representation of a certain AGE moiety need to be verified on the level of the abundance of corresponding protein, i.e. the abundance of specific modification sites need to be considered together with the data on gene expression on the RNA and protein levels. Moreover, the changes in protein degradation rates need to be taken into account.

As was demonstrated in the pioneer study of Bechtold and co-workers, stress-related up-regulation of early glycation products is much stronger in comparison to AGEs [16], which was confirmed on the level of individual glycation sites. Thus, stress conditions lead to the considerable increase of the contents of Amadori and Heyns adducts, while the response on the level of advanced glycation is much lower. Moreover, stress-specific AGEs are dominated by  $\alpha$ -dicarbonyl-derived products, and only few could originate from Amadori or Heyns products. The negligible role of early glycation products in AGE formation additionally supports the existence of powerful Amadori/Heyns product-degrading enzymatic mechanisms in plant tissues. Moreover, it might indicate the presence of a potent stress-inducible anti-glycation system scavenging or/and reducing  $\alpha$ -dicarbonyls. This assumption can be supported by the absence of changes in the carbonylated proteome, as well as glyoxal and methylglyoxal contents throughout the stress development. Thus, scavenging of these advanced glycation intermediates by the amino functioned metabolites might be the most probable scenario.

### 3.3. *In vitro* modeling of plant glycation reactions

Due to their high photosynthetic activity, green parts of plants are characterized with high contents of carbohydrates. Thus, for *B. napus* leaf tissue, these contents were more than 1  $\mu\text{mol/g f.w.}$  for the major sugars (glucose, fructose, sucrose, Asc and DHA) and were in the range of 10–60  $\text{nmol/g f.w.}$  for such compounds as glucose-6-phosphate, ribose and arabinose that are known to highly-reactive [84,85]. Hence, metabolic background of plant glycation differs essentially from that reported for mammals. Thus, animal tissues are rich in glucose (that is known to be one of the weakest glycation agents), while the contents of more reactive sugars are negligible [86]. This strong prevalence of one potential glycation agents dramatically affects the mammalian glycation patterns, which are strongly dominated by the protein fructosamines, i.e. glucose-derived Amadori compounds [64]. In contrast, photosynthetically active tissues are rich in trioses, tetroses and pentoses as well, that might affect the early glycation patterns. Moreover, these highly reactive carbohydrates might be much more susceptible to autoxidation, i.e. represent potent precursors of  $\alpha$ -dicarbonyls and, hence, AGEs. Obviously, the ability of certain sugars to act as a glycation agent depends not only on its tissue content but also on its ability to react with proteins. However, the reactivities of individual plant carbohydrates towards protein lysyl and arginyl residues, as well as their glycation potential, are still unknown.

To address this question, *in vitro* glycation models based on synthetic peptides were established recently [24,83]. Ideally, such model peptides are N-terminally protected and contain only one residue (lysine or arginine, typically in mid position) susceptible to glycation. Essential advantage of such systems is their relative simplicity: the products can be separated and analyzed by tandem mass spectrometry (MS<sup>n</sup>). Evaluation of peptide tandem mass spectra can be complemented by adequate methods of carbohydrate and  $\alpha$ -dicarbonyl analysis [87,88]. These peptide models can be potentially applied to the assessment of reactivity of individual glycation agents (i.e. sugars and dicarbonyls) and determination of their glycation potential. Such analyses might rely on integration of selected XICs (characteristic for individual glycation products) at specific retention times. This would give a possibility for screening of plant sugars for their ability to form certain Amadori/Heyns compounds and AGEs. This would essentially impact in understanding of plant glycation pathways.

## 4. Conclusions

To conclude, protein glycation is a common post-translational modification in plants. Despite this, essential differences in comparison to mammalian glycation patterns were observed. Thus, glycation patterns are strongly dominated by AGEs, while the number of Amadori-modified lysyl residues is at least one order of magnitude lower compared to human plasma proteome. Moreover, individual AGE-modified sites are not represented by their Amadori/Heyns counterparts. It indicates autooxidation of free sugars rather than glycooxidation (i.e. AGE formation from early glycated products – one of the main glycation mechanisms in mammals) as the major pathway of advanced glycation in plants. Environmental stress considerably affects glycation patterns, mostly on the quantitative level. However, due to the high heterogeneity of potential plant glycation agents, a high variability of glycation pathways and mechanisms can be expected. To clarify these pathways, simple *in vitro* models based on synthetic peptides can be used.

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# Stress-Induced Proteins in Recalcitrant Seeds During Deep Dormancy and Early Germination

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Marina I. Azarkovich

Additional information is available at the end of the chapter

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## Abstract

The role and functions of dehydrins and heat shock proteins in seeds (especially in desiccation-sensitive recalcitrant seeds) are discussed.

During the periods of dehydration, a wide variety of plants can express dehydration proteins (dehydrins), which are also members of the plant late embryogenesis abundant (LEA) protein family. Dehydrins have been most extensively studied in relation to drought and cold stresses. Dehydrins are synthesized in orthodox seeds, and their development at the final stage is associated with genetically determined seeds drying. Dehydrins amount can reach 4% of total cell proteins. At the same time, dehydrins are found in desiccation-sensitive recalcitrant seeds. The following are the functions of dehydrins with experimental evidence: binding to water and ions, binding to phospholipids, radical scavenging, phosphorylation, binding to calcium, protection of enzymes, binding to cytoskeletons, and binding to nucleic acids.

It seems evident that, in the embryo cells, heat shock induced changes in gene expression and HSP synthesis but did not result in translational discrimination of mRNAs for non-heat shock proteins. Such specific feature has been observed earlier for orthodox seeds during their development and early stages of germination. It is suggested that such response to HS is characteristic just of embryo tissues; it could be considered an additional molecular mechanism improving embryo tolerance to unfavorable environmental conditions.

**Keywords:** Recalcitrant seeds, temperature stress, dehydrins, heat shock proteins

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## 1. Introduction

Plant seeds are a unique object for studying the mechanisms of tolerance and adaptation to abiotic stresses. The seeds could not escape unfavorable environmental conditions but must

adapt to overcome them, to retain a capability of germination and to fulfill their physiological destination, that is, species preservation and distribution. Furthermore, in seeds the developmental program of the individual plant is switched over from embryogenesis to germination; in the periods of seed development and germination, seed embryos, being subjected to the action of unfavorable conditions, must change cell activity on the level of gene expression and induce the synthesis of anti-stress proteins to protect themselves and overcome stress effects. On the other hand, embryos have to provide expression of genes for proteins required for further development, that is, germination *per se*.

Different environmental stresses to a plant may result in similar responses at the cellular and molecular level. This is due to the fact that the impacts of the stressors trigger similar strains and downstream signal transduction chains. Stressors such as drought (lack of environmental water), salinity (high osmolarity), and cold, especially frost (lack of liquid water), induce water deficiency [1]. All three forms of abiotic stress affect the water relations of a plant on the cellular as well as whole plant level, causing specific as well as unspecific reactions, damages and adaptation reactions. The stabilizing effect of liquid water on the membrane can be supported by compatible solutes and special proteins. At the metabolic level, osmotic adjustment by synthesis of low-molecular osmolytes (carbohydrates, betains, proline) can counteract cellular dehydration and turgor loss [1].

During the periods of dehydration, a wide variety of plants can express dehydration proteins (dehydrins), which are also members of the plant late embryogenesis abundant (LEA) protein family. For the first time, LEA proteins were characterized in ripening seeds [2]. But they are widely investigated now [3–5]. However, their precise role has not been clear yet. These proteins are supposed to protect cells from water loss; they can behave as molecular chaperones [2]. Now the expression of LEA proteins has been shown not only in ripen seeds but also in other plant (or animal) tissues. LEA proteins are induced by osmotic or cold stress, by exogenous ABA (abscisic acid) [6]. It is believed that LEA proteins are non-catalytic proteins. Practically, all LEA proteins are water-soluble hydrophilic heat-stable and unfolded proteins. LEA proteins are synthesized in orthodox seeds, and the development of which at the final stage is associated with genetically determined drying. Their amount can reach 4% of total cell proteins [7]. At the same time, LEA proteins are found in desiccation-sensitive recalcitrant seeds. That is why the study of recalcitrant seeds is of great interest. On the one hand, these seeds produce LEA proteins, and on the other hand, they are sensitive to desiccation. Therefore, a characterization of LEA proteins in recalcitrant seeds can help understanding their role, for example when plant cells are challenged by abiotic stress as cold or salt stress.

LEA proteins are classified into more than seven distinct groups [8]. Among the induced LEA proteins, dehydrins (group II of late embryogenesis abundant proteins) have been most commonly studied, but our knowledge of their fundamental role in the cell is incomplete.

## 2. Dehydrins—stress-induced proteins

The accumulation of dehydrin transcripts and proteins during dehydration and a correlation between the level of drought tolerance and the amount of dehydrin present strongly suggest

that they are involved in protecting the plant from the negative effects of dehydration [9–11]. In recent years, dehydrins are studied very intensively, which resulted in the appearance of numerous reviews and research papers [12–18].

The dehydrins are very hydrophilic proteins and exhibit an unusually low level of recognizable structure [10, 19]. The main characteristic of the dehydrins is the presence of one or more lysine-rich stretches of 15 amino acids (the sequence EKKGIMKIKEKLPG), called the K motifs (or K-segments), that are predicted to form class A amphipathic  $\alpha$ -helices [20, 21]. All dehydrins investigated have K-segment; it is usually used for preparing anti-dehydrin antibodies [12, 13]. The K-segment occurs 1–12 times, with 1 or 2 repeats being the most common. Since the K-segments can form amphipathic  $\alpha$ -helices, they may stabilize membranes against dehydration. Dehydrins can also contain two other motifs: an N-terminal Y segment (consensus V/TDE/QYGNP) and a serine-rich S segment [11]. The Y-segment is similar to the nucleotide-binding domain found in bacteria. Typically, 1–3 Y-segments are present at the N-terminus of a dehydrin [11]. The S-segment contains a tract of Ser residues and is present in one or no copies in a dehydrin. Dehydrins extracted from drought-stressed plants are phosphorylated on these serines [22]. The role of phosphorylation is not clear but may be correlated with translocation of dehydrins to the nucleus [23] or the increased negative charge could enhance the ability of the protein to bind divalent cations such as zinc. It has been proposed that the short amphipathic K segments of dehydrin polypeptides interact with solvent-exposed hydrophobic patches on proteins undergoing partial denaturation and thereby interfere with protein aggregate formation [10]. Amphipathic K helices could also be involved in binding membrane lipids and thus could play a more specific role in protecting lipoproteins, proteins located in membranes, and/or the membrane structure itself [10, 24].

According to the presence of the K-, S-, and Y-segments, dehydrins can be divided into five structural subgroups: Kn, SKn, KnS, YnKn, and YnSKn [10, 11]. Although not specifically included in the YSK naming system, dehydrins also contain  $\Phi$ -segments, which are rich in Gly, Thr, and many other polar amino acids. This poorly conserved segment tends to be located between the Y-, S-, and K-segments.

Dehydrins are evolutionarily conserved among photosynthetic organisms including angiosperms, gymnosperms, ferns, mosses, liverworts, algae and cyanobacteria, as well as in some non-photosynthetic organisms such as yeast [17, 25]. Dehydrins seem to be very ancient proteins—a 40-kD protein was observed in *Calothrix* sp. strain PCC 7601, and in *Nostoc* sp. strain Mac-R2, an osmotic-induced doublet at 39 and 40 kD was observed. It appears that cyanobacteria produce a dehydrin-like protein under osmotic stress [25].

The expression of many dehydrins increases by the phytohormone abscisic acid (ABA), they are also referred as RAB proteins (Responsive to ABA) [26–28]. On the other hand, protein level of some dehydrins is regulated by low temperature only [22]. In particular, studies of stress-induced accumulation of five dehydrins in *Arabidopsis* revealed that two of them (LTI30 and COR47) accumulated primarily in response to low temperature. The level of another two proteins (ERD14 and LTI29) was upregulated by ABA and low temperatures, whereas RAB18 was only found in ABA-treated plants [22]. Borovsky and coworkers [15] have found that not only cold but also drought, freezing, and exogenous ABA treatment also result in accumulation

of heat-stable dehydrin-like proteins in plant mitochondria. The most tolerant winter wheat and rye accumulate more of the heat-stable dehydrins than maize. Cold-induced accumulation of the heat-stable mitochondrial dehydrin-like proteins in all species studied was accompanied by increasing of plant cryotolerance [15].

Dehydrins have been most extensively studied in relation to drought and cold stresses [29–35]. Some experimental studies provide evidence that dehydrins contribute to freezing stress tolerance in plants and suggest that this could be partly due to their protective effect on membranes [36]. Dehydrins stabilize plant plasma and organellar membranes in conditions of stress, and further zinc may be an important co-factor in stabilization [37, 38].

The hypothesis that dehydrins have detergent and chaperone-like properties and may interact with compatible solutes to serve as structural stabilizers of macromolecules under conditions of water deficit [10] is now experimentally evident [17, 39].

Numerous *in vitro* functions have been described and proposed for dehydrins, including cryoprotection of lactate dehydrogenase (LDH), cryoprotection of purified protoplasts and chloroplasts, prevention of water loss, binding of excess ions, binding of nucleic acids, prevention of protein aggregation at elevated temperatures, and prevention of ice crystal growth. RAB18 ( $Y_2SK_2$ ) accumulates in response to the phytohormone abscisic acid (ABA), drought, and low temperature [22]. LTI29 and COR47 ( $SK_3$ ) accumulate primarily in response to low temperature but also to ABA and salt stress [22]. LTI30 ( $K_6$ ) accumulates mainly under cold stress [22]. ERD14 ( $SK_2$ ) is present in non-stressed plants although the protein level is upregulated by stress, particularly drought stress [22]. Dehydrin XERO ( $YSK_2$ ) mRNA has been found to be constitutively expressed [39].

Of these functions, the most extensively studied has been the cryoprotection of LDH, where it has been shown that dehydrins are more effective than small molecules such as sucrose at protecting LDH activity from freeze-thaw damage [39].

The following are the functions of dehydrins with experimental evidence: binding to water and ions, binding to phospholipids, radical scavenging, phosphorylation, binding to calcium, protection of enzymes, binding to cytoskeletons, and binding to nucleic acids [17].

DHN genes are also expressed significantly in seeds toward the end of maturation, a period when the seed undergoes a developmentally programmed reduction in water content [22, 40]. The LEA/dehydrin proteins have been estimated to comprise up to 4% of the total seed protein [7].

### 3. Dehydrins in orthodox and recalcitrant seeds

The categories “orthodox” and “recalcitrant” seeds are used to describe the storage behavior of seeds. Orthodox seeds undergo maturation drying and are shed from the parent plant at low moisture contents. During maturation, they acquire desiccation tolerance, allowing them to be dried without irreversible damage. Because of this ability, seeds can be stored for long



periods in cold and dry vaults. On the other hand, recalcitrant seeds do not undergo maturation drying and are shed at relatively high moisture contents.

It is believed that, in orthodox seeds, dehydrins favor the development of tolerance to osmotic stress at seed dehydration during their maturation [2, 6].

The lack of resistance of recalcitrant seeds to drying was thought to be the result of the absence of dehydrins [9, 41]. Subsequent studies, however, demonstrated that dehydrins are present in the fraction of heat-stable proteins in recalcitrant seeds of many woody species of the temperate climatic zone [41–43], including those of horse chestnut, but they were not found in species inhabiting humid tropics. This raises the question of the function performed by dehydrins in recalcitrant seeds.

In this connection, the investigation of dehydrin functions, properties, and distribution in recalcitrant seeds becomes actual. The data available so far indicate that dehydrins are present in some but not all recalcitrant seed species. They appear in response to low-temperature stress, an increase in the ABA content, and natural or artificial limited dehydration [42, 44–46].

Two tropical recalcitrant species exhibited a differential capacity to produce dehydrin-related proteins during seed maturation [43]. Dehydrins were present in axes and cotyledons of *Castanospermum australe* seeds during mid-maturation and at maturity. However, in *Trichilia dregeana*, no dehydrin-related polypeptides were detected in the mature seed. During the development of *C. australe* seeds, the nature of the dehydrin-related polypeptides accumulated in the cotyledons and axis changed and new polypeptides were detected in the mature seeds. The dehydrins present in cotyledons of mature seeds (31, 37, and 40 kDa) were still detectable after germination (i.e., in untreated seedlings) [43].

Kalembe and Pukacka [47] have compared mature and dried seeds from three species of the *Acer* genus, which differed in desiccation tolerance. Seeds of three *Acer* species—*Acer platanoides* L. (Norway maple, orthodox), *Acer pseudoplatanus* L. (sycamore, recalcitrant), and *Acer saccharinum* (silver maple, recalcitrant)—harvested during various cropping years were compared and analyzed to determine whether a genetic or an environmental influence dominated the regulation of dehydrin protein expression. The authors compared the appearance of dehydrins and small heat shock proteins in seedlots originating from cropping years that differed in weather conditions, which were monitored in detail during seed development. The experiments showed that three main dehydrins with approximate molecular weights of 46, 35, and 23 kDa were characteristic of all examined *Acer* species seeds. The three proteins were present in two seedlots of the orthodox Norway maple seeds and were noted either individually or together in all seedlots of recalcitrant *Acer* seeds. The modulation of dehydrin expression by environmental factors such as developmental heat sum and rainfall is supposed [47].

The presence of dehydrins alone in recalcitrant seeds is not sufficient to prevent desiccation injury [42, 9].

In two papers published recently [48, 49], the authors suggest an interesting point of view and “paradigm change”: LEA proteins are synthesized as response to drought stress, which takes place at the end of maturation (of orthodox seeds). But it is known that dehydrins/LEA proteins

are synthesized before maturation drying [6]. And maturation drying is genetically determined in orthodox seeds. During embryogenesis and maturation drying of orthodox seeds, dehydrins are synthesized and seeds may survive dry storage. "Typical" recalcitrant seeds [48, 49] such as *Avicennia* and *Bruguiera* have no dehydrins and high water content and they do not survive water loss during storage. Intermediate seeds (*Coffea*, *Barringtonia*) have no dehydrins during embryogenesis but have genes of dehydrins and may synthesize dehydrins after partial water loss in storage, so these seeds may survive [48, 49]. And "atypical" recalcitrant seeds (*Camellia*, *Castanea*, *Euterpe*, *Quercus*) accumulate some dehydrins at the end of embryogenesis and undergo a weak maturation drying and can be stored for a limited time [48, 49]. So, the role of dehydrins in recalcitrant seeds is not clear, and further comprehensive studies are required.

#### 4. Dehydrins in horse chestnut seeds during dormancy and germination

The mature horse chestnut (*Aesculus hippocastanum* L.) seeds are not tolerant to dehydration (i.e., belong to the recalcitrant seed type), but they are resistant to long cold stress, for instance, during winter in the central Russia when seeds are under the snow cover. The second biological peculiarity of horse chestnut seeds is that they are in the state of deep dormancy, that is, they are incapable of germinating under favorable conditions without prior prolonged incubation at low above-zero temperatures and high water content, known as stratification.

The analysis of horse chestnut seed proteins made it possible to reveal a number of unique characteristics of their proteome, which distinguish recalcitrant horse chestnut seeds from the majority of orthodox seeds and is evidently related to the specific features of their physiological behavior. These characteristics include extremely low content of globulins, predominance of water-soluble proteins located in the cytosol, and the high level of non-compartmentalized heat-stable proteins [50, 52].

The presence of heat-stable proteins (i.e., proteins resistant to high-temperature denaturation) in horse chestnut seeds is the most interesting feature of the object of our study. The results obtained demonstrated that heat-stable proteins accumulate during maturation of horse chestnut seeds and are present in freshly picked seeds in considerable amounts. These proteins account for more than 30% of soluble cytosolic proteins of the axial organs and most of soluble proteins of cotyledons (more than 80%). It is possible that in some seeds certain heat-stable proteins may function as storage deposits, being a source of nitrogen for the seedling. The involvement of heat-stable proteins in some other specific functions related to their extreme temperature resistance, characteristic amino acid composition, and high cellular content cannot be ruled out.

In order to identify and characterize stress-induced dehydrin-like polypeptides in mature recalcitrant horse chestnut seeds, we analyzed the fraction of cytosolic heat-stable proteins isolated in the period of seed dormancy and germination [52]. In our experiments, in tissues of dormant seeds, dehydrin was identified by immunoblotting as a single bright band of the polypeptide with a mol wt of about 50 kD. During radicle emergence, not only the fraction of

heat-stable proteins was reduced but also the proportion of dehydrins in it decreased. Apparently, recalcitrant seed germination is accompanied by dehydrin disappearance, like this occurs during orthodox seed germination.

Since horse chestnut seeds contain along with heat-stable proteins numerous heat-sensitive proteins, it was of importance to elucidate whether dehydrin-like proteins are present among the latter. Nobody analyzed this protein fraction for the presence of dehydrin-like proteins. In the fraction of heat-sensitive proteins of horse chestnut seeds, we detected a component with a mol wt of 80 kD, which cross-reacted with anti-dehydrin antibody, that is, it was immunologically revealed as dehydrin-like protein. The analysis of different subcellular protein fractions of axial organs, cotyledons, and cotyledon petioles showed that both 50-kD dehydrin and 80-kD dehydrin-like protein could be detected in the total homogenate protein. It seems important that one of the axis heat-sensitive polypeptides cross-reacted with the anti-dehydrin antibody but differed from heat-stable dehydrin by a higher molecular weight (about 80 kD). This is the first indication on the possible presence of dehydrin-like proteins among heat-sensitive polypeptides of horse chestnut seeds [52].

During stratification, there were no substantial changes in the content of 50-kD dehydrin. However, this protein disappeared rapidly during seed germination. Since a small heat shock protein ubiquitin plays an important role in cell protein degradation, it was of interest to elucidate whether there is any connection between dehydrin, which should disappear during seed germination, and ubiquitin, which marks specifically proteins destined for degradation. It turned out that 50-kD dehydrin cross-reacted with anti-ubiquitin antibody. This means that dehydrin ubiquitination might provide for dehydrin rapid disappearance after radicle protrusion. This fact seems very interesting because just 50-kD dehydrin disappeared firstly during horse chestnut seed germination.

Earlier we have established that embryo axes of dormant seeds are not in the dormant state and could germinate *in vitro* (72 h on water at 28 °C) in each period of stratification of dormant seeds [53]. Like during seed germination *in vivo*, 50-kD dehydrin was not detected in such axes germinated *in vitro*. As we have demonstrated [53], treatment of excised axes with ABA ( $10^{-5}$  M), cycloheximide, or  $\alpha$ -amanitin suppressed their germination. Under these conditions of suppressed growth, 50-kD dehydrin remained in the axes and was easily detected by immunoblotting. The mechanism of ABA inhibitory action on excised axis germination remains unknown. It is also unknown whether ABA induces 50-kD dehydrin synthesis in axes or simply prevents its degradation, resulting in its presence in axes on the level detected before germination. However, at the comparison of ABA-treated axes with those treated with cycloheximide, or  $\alpha$ -amanitin, the substantially stronger signal may be noted in the case of ABA treatment. This may indicate indirectly on the induction of dehydrin synthesis at germination inhibition by ABA [52].

The molecular weight of the single dehydrin we detected in the horse chestnut seeds was slightly above 50 kD. At the same time, other researchers reported other values [41, 42], for example, 12, 14, and 18 kD or 30–55 kD. So far it is difficult to explain such differences in dehydrin sizes. It is not excluded that this is related to some specific feature of horse chestnut plants or their populations in different countries. It seems more likely that these differences

are related to the influence of different growth conditions. Thus, seeds collected in different years differed in the size of dehydrins: 12, 14, and 18 kD in seeds collected in 1992; 14 kD in seeds collected in 1993, and 23, 30, and 35–55 kD in seeds collected in 1994 [41, 42].

However, dehydrins comprise only a small part of heat-stable proteins of recalcitrant seeds. The functions of other heat-stable proteins accumulating in horse chestnut seeds in great amounts during dormancy and germination, the reasons for their extreme heat resistance, and their relation to the low-temperature action during stratification remain unclear. These questions are of great interest and require further study.

We believe that the presence of the proteome of horse chestnut seeds of hydrophilic proteins capable of holding moisture may be related to the recalcitrant character of these seeds. Heat-stable proteins also promote the resistance of highly watered seed to cold stress under the conditions of stratification, thereby keeping the embryo viable. From our point of view, the mere presence of a large amount of heat-stable proteins in dormant recalcitrant horse chestnut seeds is of great interest.

## 5. Heat shock proteins

Another interesting family of stress-induced proteins is the heat shock family [54, 55].

It is known that heat stress may inhibit growth and development of plants. This may be due to heat stress itself or due to formation of ROS and other oxidants induced by heat stress. Heating also changes the structure of proteins, up to complete denaturation, and alters the activity of many enzymes. Disturbances in membranes structure, in their permeability and fluidity may cause partial or total disintegration of cells. Heat shock, that is, a short-term increase in temperature by 8–10 °C above the optimum one, is well known to induce rapid transient and reversible changes in gene expression in all living organisms. These changes result in the synthesis of specific group of polypeptides called heat shock proteins and suppression (complete or partial) of the synthesis of "normal" cell proteins synthesized by the cells before heat shock. This general biological phenomenon was qualified as a response to heat shock [55, 56]. The universality and conserved character of this response indicate its importance in cell physiology. Since heat shock proteins accumulation at heat shock in the cells of plant vegetative organs and seedlings was correlated with the development of plant tolerance to subsequent action of lethal temperatures, it was suggested that the response to heat shock is a manifestation of molecular mechanisms providing cell heat tolerance [57–59].

The induction of transcription of heat shock proteins is a common phenomenon in all living organisms. These proteins are grouped in plants into five classes according to their approximate molecular weight: HSP100, HSP90, HSP70 (chaperones), HSP60 (chaperonins), small heat shock proteins (sHSPs), and ubiquitin (8.5 kD). Higher plants have at least 20 sHSPs and there might be 40 kinds of these sHSPs in one plant species. All of the major HSPs (that is, those expressed in very high amounts in response to heat and other stresses) have related functions: they ameliorate problems caused by protein misfolding and aggregation. However, each major

HSP family has a unique mechanism of action. Some promote the degradation of misfolded proteins (ubiquitin, and various ubiquitin-conjugating enzymes); others bind to different types of folding intermediates and prevent them from aggregating (HSP70 and HSP60); and still another (HSP100) promotes the reactivation of proteins that have already aggregated [55].

During two recent decades occurred after heat shock proteins discovery, the notions concerning their properties and role gradually widened and became much more complex. Now, we know that many heat shock proteins are molecular chaperons and facilitate protein-protein interactions in the cell, that heat shock proteins can be present in normal cells not subjected to stress, that they can be expressed at some developmental stages in the absence of heat shock, and that their synthesis can be induced by other stress types [57, 60–62]. Changes in gene expression resulting in heat shock proteins accumulation in the cells are evidently play an important physiological role and somehow protect cell structures and separate protein components against injuries induced by various stressors and increase cell tolerance and their adaptation to unfavorable environmental conditions [63]. Nevertheless, so far we did not decipher completely heat shock proteins functions and molecular mechanisms of their action. The possible heat shock effects on the synthesis of normal non-heat-shock proteins in various plant tissues are still less studied; it is not clear whether these effects are universal to the same degree as those of heat shock proteins gene expression. In many cases, heat shock suppressed the total protein synthesis and especially that of non-heat-shock proteins. This effect was evidently controlled on the level of translation because the normal pattern of protein synthesis was rapidly restored after the change in the temperature and transcription suppression with  $\alpha$ -amanitin. It is known that heat shock inhibited total protein synthesis in the vegetative organs of seedlings and plant cell cultures. This was related not to the inhibition of non-heat-shock mRNA synthesis but to incapability of these mRNAs to be translated under heat shock conditions. However, this specific response to heat shock was not evidently universal because it was not observed in seed embryos during seed development and germination. Thus, it has been shown for soybean and common bean seeds that the synthesis of storage proteins and many other non-heat shock proteins and their mRNAs was not reduced and even was activated under heat shock and occurred along with the synthesis of heat shock proteins [64, 65]. In embryos of wheat [66], sorghum [67], maize [68], and pea [69], heat shock markedly activated proteinsynthesisduringearly stages of germination, and heat shock proteinsynthesis was induced simultaneously with the synthesis of the bulk of proteins produced by embryo tissues before heat shock. On the basis of these facts, it was concluded that such a specific response of protein synthesis to heat shock observed in seed embryos of many grasses during seed development and germination could have a definite physiological significance; this could be a manifestation of additional molecular mechanisms improving embryo tolerance to unfavorable environmental conditions and, as a consequence, their viability [69].

Small heat shock proteins function as intracellular chaperones for other proteins. They play an important role in protein-protein interactions such as folding. They assist in the establishment of proper protein conformation and prevent unwanted protein aggregation. By helping to stabilize partially unfolded proteins, HSPs aid in transporting proteins across membranes within the cell [57]. HSPs recognize and bind to other proteins when these other proteins are in non-native conformations. The non-native conformations of these proteins could be due to protein-denaturing stress or due to immature peptides folded, assembled, or localized to an

appropriate cellular compartment. In the presence of ATP at normal physiological concentrations, sHSPs change their conformation and releases denatured protein, allowing other molecular chaperones such as HSP70 to renature the protein and renew its biological activity. In the absence of ATP, sHSPs such as  $\alpha$ -crystallin are more efficient than HSP70 in preventing stress-induced protein aggregation. *In vitro*, sHSPs selectively bind and stabilize proteins and prevent their aggregation at elevated temperatures in an ATP-independent way and protect enzymes against heat-induced inactivation.

In recalcitrant of chestnut (*Castanea sativa*) seeds, a 20-kD protein has been purified from cotyledons, where it accumulates at levels comparable to those of major seed storage proteins [58]. This protein, termed Cs sHSP 1, forms homododecameric complexes under non-denaturing conditions and appears to be homologous to cytosolic class I small heat shock proteins (sHSPs) from plant sources. *In vitro* evidence has been obtained that the isolated protein can function as a molecular chaperone; it not only increases, at stoichiometric levels, the renaturation yields of chemically denatured citrate synthase but also prevents the irreversible thermal inactivation of this enzyme. Although a role in desiccation tolerance has been hypothesized for seed sHSPs, this does not seem to be the case for Cs sHSP 1. The presence of immunologically related proteins in orthodox and recalcitrant seeds of 13 woody species has been investigated [58]. The results indicate that the presence of Cs sHSP 1- like proteins, even at high levels, is not enough to confer desiccation tolerance and that the amount of these proteins does not furnish a reliable criterion to identify desiccation-sensitive seeds. Additional proteins or mechanisms appear necessary to keep the viability of orthodox seeds upon shedding. The synthesis of small HSPs may be an important part of seed developmental program [61]. As detected in pea [70], and in other species, the class I sHSPs accumulate in *Arabidopsis* seeds at midmaturation and decline during germination [61]. The similarity of sHSP regulation in such diverse species supports the conclusion that there is a selective advantage to this pattern of sHSP accumulation. The correlation of sHSP expression with the development of desiccation tolerance and dormancy suggests a possible role for the sHSPs in either or both of these processes, as has been previously hypothesized [62, 71].

It was found that one major small heat shock protein existed with a molecular mass of 22 kDa and was detectable at high concentrations in seeds of three *Acer* species (orthodox and recalcitrant). After the seeds were dried, the content of this protein significantly increased. The largest content of this protein was observed in the oldest seeds, especially in embryonic axes. The proteins identified may play a protective role during water deficit and storage [47].

## 6. Heat shock proteins in recalcitrant horse chestnut seeds

In the work [72], first data are obtained about functioning of molecular mechanisms providing for perception and transduction of heat signal and inducing heat shock proteins synthesis in the cells of embryo axes of dormant recalcitrant seeds, which are in metabolically active state but could not germinate. Embryo axes, cotyledon pieces, and cotyledon petioles were excised from recalcitrant horse chestnut (*Aesculus hippocastanum* L.) seeds in different times after the

start of cold stratification and incubated at 28 or 40 °C on the medium containing <sup>35</sup>S-methionine and 50 µg/ml chloramphenicol for 4 h. The total rate of protein synthesis, the pattern of synthesized polypeptides, their distribution between subcellular fractions, and their relation to heat denaturing (5°min at 75 °C) were assessed. It was established that in all embryo parts, especially in axes, heat shock markedly activated protein synthesis in the beginning of stratification and to a lesser degree after ten weeks of stratification; heat shock suppressed protein synthesis at radicle emersion and especially during axial organ growth. Independently of the duration of stratification, which gradually released seed deep dormancy, isolated axes, cotyledons, and cotyledon petioles synthesized *in vivo* numerous diverse polypeptides at both 28 and 40°C. Newly synthesized polypeptides were present in the fractions of cell structures and cytosol; they differed in molecular weights, the intensity of labeling, and tolerance to heat denaturing. None of the dominating polypeptides present initially in all embryo parts and belonging mainly to heat-stable proteins was synthesized either at 28°C or 40°C. Some proteins synthesized at 40°C could be considered heat shock proteins because they were not synthesized at 28°C or their synthesis was markedly activated by heat shock. No less than 10 proteins behaved as obvious heat shock proteins; they were predominantly heat-sensitive soluble cytosolic proteins. All heat shock proteins, except those with mol wts of 220 and 34 kD, were highly labeled proteins. Some of them were characteristic of cell structures (220, 90, 20, and 18 kD); others were detected only in the cytosol (100, 80, and 34 kD). The synthesis of heat shock proteins did not depend on transcription and occurred on pre-existing mRNAs. An embryo capability of responding to heat shock did not depend on the seed physiological state and their germinability; it was similarly manifested in stratified and non-stratified seeds [72].

The analysis of heat shock action on gene expression in embryo tissues in dormant horse chestnut seeds demonstrated translation activation leading to the induction of a wide set of heat shock protein synthesis at the maintenance of the bulk of normal (non-shock) cellular protein synthesis. Due to this specific embryo response to heat shock, at early stages of germination and even under unfavorable conditions, embryo cells retain a capability of continuation or supporting on the sufficient level of the synthesis of proteins required for cell activity switching over to new developmental program, from embryogenesis to germination, and thus increase the reliability of germination. We believe that the absence of discrimination of non-heat shock mRNA translation during heat shock is specific to embryo tissues and could be considered an additional mechanism facilitating seed adaptation to unfavorable environmental conditions and successful germination.

Horse chestnut seeds survive successfully (and even require for deep dormancy release) the period of long cooling (18–22 weeks), retaining the high water content in their cells, and thus they are well adapted to overcome or correct damages arising under these conditions. However, according to current knowledge, heat shock proteins just fulfill this protective function. Therefore, it might be that, in mature dormant horse chestnut seeds, some amounts of required heat shock proteins are already present. These heat shock proteins could be synthesized and accumulated under the influence of elevated temperatures in the embryo cells during seed development or after their falling, that is, in response to heat shock, and they were preserved in the cells after seed entry into deep dormancy in the metabolically active state;



they could improve embryo tolerance to unfavorable environmental conditions during stratification and thus increase seed viability.

We believe that the absence of discrimination of non-heat shock mRNA translation during heat shock is specific to embryo tissues and could be considered an additional mechanism facilitating seed adaptation to unfavorable environmental conditions and successful germination.

Induction of heat shock proteins synthesis is a universal feature of the response to heat shock. According to our data, all tissues isolated from the embryos of dormant recalcitrant horse chestnut seeds responded to heat shock not only by continuation of non-heat shock protein synthesis but also by induction of similar sets of heat shock proteins. Two observations are of interest. We did not observe any dramatic changes in the set of heat shock proteins synthesized by isolated axes in the response to heat shock in the course of stratification, which evidently facilitate seed deep dormancy release. Moreover, heat shock proteins synthesis was readily detected in axes excised from non-stratified seeds, that is, it did not depend on seed capability of germination. This indicates independence of heat shock proteins synthesis at heat shock in the course of stratification of the embryo physiological state and its capability of germination. At the same time, some of our data indicate that stratification still somehow affected embryo physiological state. Thus, in the course of stratification, sensitivity of isolated axis growth to abscisic acid and indol-3-acetic acid decreased [53], some characteristics of the proteome changed [50], and sensitivity of isolated axis translation to heat shock changed as well. However, this did not affect embryo tissue capacity to respond to heat shock. It is likely that signals providing for dormancy state, its release, and seed germination do not interact with signals leading to heat shock proteins synthesis induction. Furthermore, heat shock proteins' gene expression in isolated axes of dormant recalcitrant horse chestnut seeds was not dependent on transcription and was controlled predominantly on the level of translation. This means that all components required for the complex molecular mechanism of heat shock proteins gene expression were present in axis cells of mature seeds and were evidently produced still during seed development, may be under the influence of elevated temperatures. After mature seed falling, this mechanism is retained in the cells in the functionally active state and is capable of a rapid initiation of heat shock proteins synthesis in response to heat shock or another stress. However, the realization of this mechanism of heat shock proteins accumulation under natural conditions of stratification seems not very probable because the rate of protein synthesis under low temperature is low and heat signal is absent. Nevertheless, horse chestnut seeds survive successfully the period of long cooling (18–22 weeks), retaining the high water content in their cells and thus they are well adapted to overcome or correct damages arising under these conditions. However, according to current knowledge, heat shock proteins just fulfill this protective function. Therefore, it might be that, in mature dormant horse chestnut seeds, some amounts of required heat shock proteins are already present. These heat shock proteins could be synthesized and accumulated under the influence of elevated temperatures in the embryo cells during seed development or after their falling, that is, in response to heat shock, and they were preserved in the cells after seed entry into deep dormancy in metabolically active state; they could improve embryo tolerance to unfavorable environmental conditions during stratification and thus increase seed viability. This suggestion is supported by our observation



that one of heat shock proteins, ubiquitin, was present in dormant horse chestnut seeds in functionally active state (i.e., in association with dehydrins) [52].

## 7. Conclusions

Plants cannot avoid the exposure to different abiotic factors but adapt morphologically and physiologically by some other mechanisms. Almost all stresses induce the production of groups of proteins called dehydrins and heat shock proteins (HSPs), which comprise several evolutionarily conserved protein families. Accumulation of dehydrins can be induced not only by drought but also by cold, salinity, and treatment with abscisic acid.

Since HSPs accumulation at heat shock in the cells of plant vegetative organs and seedlings was correlated with the development of plant tolerance to subsequent action of lethal temperatures, it was suggested that the response to heat shock is a manifestation of molecular mechanisms providing cell heat tolerance.

Heat stress induces the known genes for HSPs and chaperones: *hspA*, *groES*, *groEL1*, *groEL2*, *dnaJ*, *hspG*, *dnaK2*, *clpB1*, and *htrA* for protease, *sigB* for the  $\sigma$ -factor of RNA polymerase, *hik34* for sensory histidine kinase, *sodB* for superoxide dismutase, and some other genes. Using DNA microarrays, it has been shown that none of the aforementioned genes is induced by heat stress specifically [73, 74]. Expression of these genes is induced by high osmolarity, NaCl, oxidative stress ( $H_2O_2$ ), high light, and UV-B. This phenomenon has been observed earlier. However, before the application of DNA microarrays, the information on HSPs that respond to various stresses has been fragmented and limited to studies of individual genes. Now it is clear that the genes whose transcription is specifically induced by high temperatures are classified as unknown. The entire list of genes of the genuine HSPs is limited to following titles: *sll0441*, *sll0688*, *sll1106*, *sll1884*, *slr0852*, *slr0095*, and *slr1597* [75]. The remaining genes that are induced by heat shock belong rather to a group of the general-stress-responsive (GSR) genes [76], and HSP may be renamed as general stress protein (GSP) [76, 77].

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## Signalling in Stress

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# Molecules and Methods for the Control of Biotic Stress Especially the Insect Pests — Present Scenario and Future Perspective

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Santosh Kumar Upadhyay and Sudhir P. Singh

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/62034>

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## Abstract

Continuous increase in population has unbalanced the demand and supply of agricultural produce. In this scenario, food security in a sustainable manner is being challenged due to several factors. Insect pests are considered as one of the major factors, which accounts for 35–100% crop damage, worldwide. Synthetic insecticides contributed significantly, but several safety concerns are associated with them. Transgenic crops with enhanced biotic or abiotic stress tolerance have shown promising contribution in achieving greater crop productivity. Transgenic cotton expressing Cry toxin of *Bacillus thuringiensis* has tremendously increased the production as well as the societal status of farmers in our country. However, a concomitant increase in the population of minor pests like aphids, whiteflies and others has demanded certain new approaches. Researchers have isolated several other toxic proteins like lectins, protease inhibitors, amylase inhibitors, chitinases, and tried various novel approaches like gene pyramiding, tissue specific expression and modulation in metabolites expression to combat emerging problems of insect pests. Conversely, the emergence of a new type of crop insect pests demands more specific effort for each insect. Besides this, there are several safety and ethical concerns that are associated with the use of genetically modified crops, which also need to be resolved as per demand. Development of a dedicated scientific forum for the proper demonstration of advantages and disadvantages of genetically modified crops to the citizens at ground level might be useful in resolving the societal and ethical concerns in our country.

**Keywords:** Insecticidal proteins, Cry toxins, Lectins, Protease inhibitors, Chitinase, Transgenic crops

## 1. Introduction

World population is projected to increase over 1,000 million in the next four decades. An immediate priority for agriculture industry is to achieve maximum production in an environmentally sustainable and cost-effective manner. Food security is on high agenda at the political and social level [1]. Our progeny can face a severe shortage of food supply due to the over demand of continuously increasing population. Jaques Diouf, the Director General FAO, stated (2011) “*The silent hunger crisis, affecting one sixth of all humanity, poses a serious risk for world peace and security*”. The current challenge is to increase primary crop production in agricultural sustainability manner. In order to achieve these goals, it is important to identify and address the major limitations of productivity. Crop damage caused by insect pests is one of the major confinements, which is estimated to be around 35–100%, globally [2]. Synthetic insecticides have made significant contributions in food production, but they are also responsible for environmental and health hazards.

Transgenic crops with enhanced biotic or abiotic stress tolerance have shown promising contribution in achieving greater food security. A milestone was established about 25 years ago with the development of genetically engineered tobacco expressing the entomotoxic Cry protein from the bacterium *Bacillus thuringiensis* (Bt) [3, 4]. Presently, a number of Bt-Cry protein containing products are in the market of the United States ([www.aphis.usda.gov](http://www.aphis.usda.gov)) and some other countries. In March 2002, the Government of India permitted the release of transgenic cotton (Bollgard) expressing Bt toxins-Cry1Ac for commercial cultivation, which conferred resistance against bollworms [5]. Subsequently, BollgardII was released, which expresses Cry2Ab toxin along with Cry1Ac. Bt-Cry proteins have increased the productivity of crops substantially by controlling the major insects of order Lepidoptera and Coleoptera [6]. However, a concomitant increase in the population of minor pests (like whiteflies, aphids, leafhoppers and others) has threatened the success of Bt-transgenic crops [7–9]. An alternative strategy is to take advantage of the plant’s own defense mechanisms, either by maneuvering the expression of the endogenous defense proteins or by introduction of an insect toxic gene (like lectins) derived from another plant. Several insecticidal proteins encoding genes have now been isolated from different sources and introduced into crop genomes to combat the issue of various groups of insect pests [10, 11]. Simultaneously, a new approach based on RNA interference is also reported for the control of crop insects [12–14]. Present chapter briefs about the insecticidal proteins and transgenic strategies for the control of crop insects.

## 2. Insecticidal proteins

### 2.1. Cry toxins of *Bacillus thuringiensis*

Introduction of Bt-Cry toxins revolutionized the area of insect control through transgenic technology. These are toxic to the insects of orders Lepidoptera, Diptera, Hymenoptera, Coleoptera and also to nematodes. These are produced as parasporal crystalline inclusions in *B. thuringiensis*. More than 500 Cry proteins/genes have been discovered till date, which are

classified into 67 groups (Cry1–Cry67) on the basis of the primary structure [15, 16]. The genes are further divided into four phylogenetically unrelated protein families with different modes of action. These are: (1) three domain Cry toxins (3D) family, (2) mosquitocidal Cry toxins (Mtx) family, (3) binary-like (Bin) family and (4) the Cyt family of toxins [17]. Some Bt strains produce an additional insecticidal toxin called as VIP (vegetative insecticidal proteins) during the vegetative growth phase. Three VIP toxins: VIP1/VIP2, a binary toxin, and VIP3 have been characterized till date [18, 19].

Several insect-resistant transgenic crops have been developed by expressing Bt-Cry proteins, among which corn, cotton, soybean and canola are the most important crops. These transgenic crops are mostly expressing the Cry1Ac and Cry2Ab to control the chewing pests like *H. armigera*, *H. zea* and *Pectinophora gossypiella*, *Heliothis virescens* and *Ostrinia nubilalis* [20]. Some other cry toxin based products are also commercialized, which express Cry1A, Cry1F, Cry1EC, Cry34Ab/Cry35Ab binary toxin, Cry1Ab and Cry3Bb for the control of lepidopteran insect *Spodoptera frugiperda*, *S. litura* and coleopteran insect *Diabrotica virgifera* [6, 20, 21]. Further detail about the commercialized insecticidal crops are given in a later section.

## 2.2. Lectins

Lectins are carbohydrate-binding proteins, which possess at least one non-catalytic domain for specific and reversible binding to mono- or oligosaccharides [22, 23]. A typical lectin is multivalent in nature, therefore agglutinate cells. Lectins are extensively distributed in nature from prokaryotes to eukaryotes. The specific interaction with glycoconjugates makes them valuable in biomedical sciences and biotechnology [24]. Carbohydrates present in viruses, microorganisms, fungi, nematodes or phytophagous insects interact with plant lectins [25, 26]. In the past decades, many plant lectins are reported to be toxic to several economically important insect pests of various orders [27–29]. To analyze the insecticidal properties under natural conditions, many transgenic plants expressing lectins have been developed. The toxic effects of different lectins have been demonstrated on several insect species; these effects range from a severe delay in development to high mortality in insects [11].

### 2.2.1. GNA-related lectins

*Galanthus nivalis* agglutinin (GNA) purified from snowdrop bulbs is the best studied plant lectin for insecticidal properties. The snowdrop lectin specifically binds to terminal mannose residues in high-mannose-N-glycans, which occur very frequently on insect glycoproteins [30]. Toxicity of GNA has been shown for a wide range of insects; but homopteran insects are highly sensitive to GNA. Several GNA-related lectins have been isolated from different *Allium* species which have shown the potential for insect control [11, 29]. Further, accumulation of some lectins like *Allium porrum* agglutinin in the phloem sap in natural situations support the defensive role of lectins against sap-sucking insects [31].

GNA and related lectins have been successfully expressed for resistance against insect pests into a variety of crops [32]. Transgenic rice expressing ASAL caused significant mortality in nymph of hemipteran insect pests [33]. Onion (*Allium cepa*) lectin has shown more potential

against mustard aphid (*Lipaphis erysimi*) in comparison to GNA and ASAL (*Allium sativum* leaf agglutinin) [34]. Transgenic rice expressing ASAL exhibited protection against tungro disease also, after infestation with the *N. virescens* [35]. Vajhala et al. [36] recently demonstrated significant protection in ASAL expressing transgenic cotton against jassid and whitefly. ASAL is also reported to be toxic to chewing insects like *Helicoverpa armigera* and *Spodoptera litura* [27] and several other sucking insects like *Nephotettix virescens* and *Nilaparvata lugens* [37]. Studies related to the mechanism of toxicity showed that ASAL shares the similar receptors with Bt-Cry toxin [28], but both the proteins interact at different positions without steric hindrance and increased the toxicity of each other [29]. Therefore, they can be pyramided together for broad-range insect resistance.

#### 2.2.2. Legume lectins

Legume lectins are purified from seeds and bind to carbohydrate structures like Thomsen-nouveau (Tn) antigen or complex N-glycan with terminal galactose and sialic acid residues. Pea lectin (*Pisum sativum* agglutinin, PSA) expressed in transgenic oilseed rape (*Brassica napus*) shows growth retardation of the pollen beetle larvae (*Meligethes aeneus*) [38] and no effect on the adult beetles [39]. A legume lectin known as Gleheda purified from ground ivy (*Glechoma hederacea*) exhibits high insecticidal activity against the Colorado potato beetle larvae (*Leptinotarsa decem-lineata*) [40]. GS-II lectin isolated from the seed of *Griffonia simplicifolia* shows toxicity to Cowpea weevil (*Callosobruchus maculatus*) [41]. A mannose-binding legume lectin concanavalin A (ConA) from jackbean has shown toxicity to the hemipteran pea aphid (*Acyrtosiphon pisum*) [42, 43] and tara plant hopper (*Tarophagous proserpina*) [44].

#### 2.2.3. Hevein-related lectins

Hevein-related plant lectins exhibit specificity for chitin (chitin forms endo- and exo-arthropods, nematodes and fungi). These are also studied for insecticidal properties [45]. Due to the absence of chitin in mammals, hevein-related lectins are considered safe for the usage in genetically modified crops. Wheat germ agglutinin (WGA) has shown a negative effect on the development of the cowpea weevil (*Callosobruchus maculatus*) larvae and southern corn root worm (*Diabrotica undecimpunctata*) [46, 47]. WGA is active against lepidopteran insect larvae also [47, 48].

#### 2.2.4. Other insecticidal lectins

Several other plant lectins have shown insecticidal property. Transgenic tobacco plants expressing tobacco leaf lectin (NICTABA) is detrimental to the cotton leafworm (*S. littoralis*) and the tobacco hornworm (*M. sexta*) [49]. Another protein, *phloem protein 2* (PP2) belonging to the NICTABA family, also possesses insecticidal activity [50, 51]. The amaranthins and the jacalin-related lectins have also shown the potential for insect control, especially against sap-sucking insect pests. Transgenic cotton expressing *Amaranthus caudatus* agglutinin (ACA) under the control of a phloem-specific promoter shows a strong resistance against nymphs of the cotton aphid (*Aphis gossypii*) [52]. Transgenic tobacco expressing Heltuba, a jacalin-related lectin from the *Helianthus tuberosus*, showed reduced development and fecundity of the peach-

potato aphid (*M. persicae*) [53]. Another promising jacalin-related lectin HFR1 is produced in resistant varieties of wheat (*T. aestivum*) during infestation by the Hessian fly larvae. Although HFR1 has not shown any toxicity against Hessian fly, it shows the strong insecticidal activity to the larvae of fruit fly (*D. melanogaster*) [54].

### 2.3. Proteinase inhibitors

Proteinase inhibitors (PIs) are small molecular weight proteins which affect several metabolic pathways. They are the major components in seeds and storage organs of crops. Mickel and Standish [55] demonstrated the role of PIs in plant defense for the first time and noticed the abnormality in the development of larvae of certain insects fed on soybean products. The feature was attributed to trypsin inhibitors, and it was found to be toxic to the larvae of flour beetle (*Tribolium confusum*) [56].

PIs inhibit the digestion of proteins in midgut and cause mortality of insects due to nutritional imbalance [57, 58]. PIs also interfere with several metabolic processes (like moulting) by blocking the proteolytic activation of enzymes [59]. They affect growth and development, multiplication rate and insect life span [60–62]. PIs have been expressed in several transgenic plants for resistance against insect pests of several classes [63–65]. Pea and soybean trypsin–chymotrypsin inhibitors (PsTI-2, SbBBI) belonging to the Bowman–Birk family [66] and mustard-type trypsin–chymotrypsin variant Chy8 [67] cause significant mortality of pea aphid *A. pisum*. Plant-derived PIs have been used for the development of insect-resistant transgenic plants and projected as an alternative to Bt-Cry proteins [68, 69].

The majority of plant PIs originate from three main families, namely Solanaceae, Leguminosae and Gramineae [70]. Plant PIs can be grouped into four classes: serine, thiol, metallo and aspartyl. Most plant PIs are inhibitors of microbial and animal serine proteases, such as chymotrypsin, trypsin, elastase and subtilisin [71]. Specificity of protease inhibitor families is mainly based on the amino acid residues present in the active site [72].

#### 2.3.1. Serine (Serpin) protease inhibitors

It is found in almost all kingdoms of organisms [73–76]. Several serine PIs have been purified from plants and characterized [77, 78]. Plant serine PIs show inconsistent and varied specificities towards plant proteases [79]. *Hordeum vulgare* serine PI inhibits trypsin, chymotrypsin [80], thrombin, plasma kallikrein, Factor VIIa and Factor Xa [81]. *Triticum aestivum* serine PI inhibits chymotrypsin and cathepsin G [82]. Serine protease inhibitors have been used most commonly for the development of transgenic plants for the control of insect pests [83–85].

#### 2.3.2. Cysteine protease inhibitors

An inhibitor of cysteine proteinases was first described in egg white by Sen and Whitaker [86] and was later named cystatin [87]. Cysteine proteinases inhibitors are widely distributed in plants, animals and microorganisms [88]. Their role in defense has been explored by *in vitro* analysis on inhibition of digestive proteinases from insect pests and nematodes [89–91]. First plant cystatin was isolated from rice seeds and as of now, more than 80 members of different

plant species have been characterized [92, 93]. Barley cystatin in artificial diets hampered the life cycle of two aphid species and also in transgenic *Arabidopsis* [94]. Expression of such inhibitors in maize enhanced the resistance against phytophagous mites [95]. Inhibition of these proteases provides a promising control on insects and therefore PIs can be employed as a potential source of defense in plants against insect pests.

### 2.3.3. Aspartyl protease inhibitors

It is relatively less studied class, due to the rare occurrence [91]. Potato tubers possess cathepsin D, an aspartic proteinase inhibitor which showed substantial amino acid sequence similarity with the soybean trypsin inhibitor [96]. Aspartic proteases have been found in coleoptera species, such as *Callosobruchus maculatus* [97] and *H. hampei* [98], in which the acidic pH in midgut provides a favourable condition for these proteases [58].

### 2.3.4. Metallo-proteases inhibitors

The metallo carboxypeptidase inhibitors (MCPIs) have been identified in solanaceous plants tomato and potato [99]. The MCPIs are 38–39 amino acid residues long polypeptide [100, 101]. Plants have evolved at least two families of metalloproteinase inhibitors, the metallo-carboxypeptidase inhibitor family in potato and tomato [102] and a cathepsin D inhibitor family in potato [103]. The inhibitor is produced in potato tubers and accumulates with potato inhibitor I and II families (serine proteinase inhibitors) during tuber development. The inhibitor also accumulates in potato leaf with inhibitor I and II in response to wounding and have the potential to inhibit all the major digestive enzymes (like trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B) of higher animals and many insects [104].

## 2.4. $\alpha$ -Amylase inhibitors

$\alpha$ -Amylases ( $\alpha$ -1,4-glucan-4-glucanohydrolases) are hydrolytic enzymes, which catalyze the hydrolysis of  $\alpha$ -1,4-glycosidic bonds in polysaccharides. They are present in microorganisms, animals and plants [105–107]. They are the most important digestive enzymes of many insects which feed exclusively on seed products. Inhibition of  $\alpha$ -amylase impairs the digestion in an organism and causes shortage of free sugar for energy.  $\alpha$ -Amylase inhibitors ( $\alpha$ -AI) are found in many plants as a part of the defense system and abundant in cereals and legumes [108–111].

$\alpha$ -AI of *Phaseolus vulgaris* is the most studied amylase inhibitor and have shown toxic effects to several insect pests [110, 111]. Like lectins, they possess carbohydrate-binding property. There are at least four types of *Phaseolus* amylase inhibitors on the basis of  $\alpha$ -AIs: AI-1, AI-2, AI-3 and the null type [112]. AI-1 is present in the most cultivated common bean varieties and inhibits mammalian  $\alpha$ -amylases. It also inhibits  $\alpha$ -amylases in insects like *C. chinensis*, *C. maculatus* and *B. pisorum* [106]. AI-2 is 78% homologous to AI-1 and found in few wild accessions. It inhibits the *Z. subfasciatus* larval  $\alpha$ -amylase and pea bruchid  $\alpha$ -amylase [106, 111, 113]. This inhibitor is a good example of co-evolution of insect digestive enzymes and plant defense proteins.



They are potential molecules for the development of insect-resistant transgenic plants [114, 115]. Seeds of transgenic pea and azuki, expressing  $\alpha$ -AI-1 inhibitor of *P. vulgaris*, shows resistance against pea weevil (*Bruchus pisorum*), cowpea weevil (*C. maculatus*) and azuki bean weevil (*Callosobruchus chinensis*) [110, 113, 116].

## 2.5. Chitinase

Chitinases are being employed in plant defense in many ways. It has been used in controlling the growth of fungi and insects. Expression of poplar chitinase in tomato leads to growth inhibition in Colorado potato beetle [117]. Secretome analysis of tobacco cell suspension represents chitinase as the major defense protein [118]. A chitinase-like domain containing 56-kDa defense protein (MLX56) provides strong resistance against cabbage armyworm, *Mamestra brassicae*, and Eri silkworm, *Samia ricini* [119]. Two chitinase like proteins LA-a and LA-b (latex abundant) from Mulberry (*Morus* sp.) latex are found to be toxic against *Drosophila melanogaster* [120].

Chitinases have also been isolated from insects and found to be equally promising in plant defense. Transgenic tobacco plants expressing chitinase of tobacco hornworm (*Manduca sexta*) shows resistance to tobacco budworm *Heliothis virescens* [121]. Hornworm chitinase expressing transgenic plants are also resistant against fungal infection [122]. Further, a recombinant baculovirus expressing chitinase of hard tick (*Haemaphysalis longicornis*) has been shown as bio-acaricide for tick control [123].

## 3. Insect-resistant transgenic crops

Development of many transgenic crops has been reported for insect resistance. Both private and public sector organizations are involved in the process and they used  $\delta$ -endotoxins of *Bacillus thuringiensis* to achieve resistance against insects. Among transgenic plants, cotton and maize were the most successful and released for commercial cultivation. These crops are being adopted annually at very high rates. In other words, area under Bt-crops are increasing day-by-day. Successful deployment of these crops has decreased the pesticide usage. However, the sustainability and durability of pest resistance are still a matter of discussion. It is also important to focus on next-generation insect-resistant transgenic crops.

### 3.1. First-generation insect-resistant transgenic crops

Insect-resistant transgenic crops have not only increased the economy but also the environmental and health benefits [69, 124]. Six transgenic crops (canola, corn, cotton, papaya, squash and soybean) were planted in 2003 in the USA alone. These crops increased farm income by US\$ 1.9 billion by producing an additional 2.4 million tonnes of food and fiber and reduced the use of pesticides by 21,000 tonnes.

In 2009, China government approved the cultivation of Bt-rice (the country has been growing Bt-cotton since 1997). Farm surveys of randomly selected households cultivating Bt-rice

varieties have been performed. The benefit of Bt-rice has been acknowledged to the level of small and poor farmers, it is due to the lesser crop damage by the insects and therefore higher crop yields and less use of pesticides. An improved health has also been observed in Bt-rice cultivating farmers compared to non-Bt rice cultivating farmers [126]. Government of India approved the cultivation of Bt-cotton in 2003, which resulted in a 70% reduction in insecticide applications. This saves up to US\$ 30 per ha in insecticide costs and results 80–87% increase in cotton yield [127]. A spectacular decrease in pesticide usage in Bt cotton fields has also been reported from China. The pesticide poisoning to the farmers reduced from 22% to 4.7% [128].

To assess probable hazards of Bt toxins on non-target insects, field evaluation was performed in Spain [129]. Bt-maize did not show negative impact on non-target pests. Similar numbers of cutworms and wireworms were present in Bt versus non-Bt fields. Surprisingly, higher numbers of aphids and leafhoppers were observed in Bt field.

### 3.2. Strategies for next-generation insect resistance

#### 3.2.1. Engineering of Cry toxin by domains swapping

Most of the Cry toxins share common three-domain structure in activated form [130]. Domain I gets inserted into the target membrane and forms pore; domain II is associated with receptor binding and thus determines specificity, and domain III is also involved in receptor-binding specificity. It has been demonstrated in a couple of studies that hybrid Cry toxins exhibit higher toxicity. Domain III of Cry1Ac increased the efficacy of various other Cry1 proteins in Cry1–Cry1Ac hybrid [131]. Similarly, Singh et al. (2004) developed a hybrid toxin against *Spodoptera litura*. They replaced a region in domain III of Cry1Ea toxin by 70 amino acid homologous region of Cry1Ca. Transgenic tobacco and cotton expressing hybrid gene are highly effective/toxic to all stages of larvae of *S. litura*. Another hybrid Bt gene was developed by replacing part of domain II of Cry1Ba with that of Cry2a [132]. The transgenic potato expressing the hybrid toxin showed resistance against Colorado potato beetle, potato tuber moth and European corn borer. The strategy provides new opportunities for resistance management as the target receptor recognition of hybrid toxins is expected to be different from currently used Cry toxins.

#### 3.2.2. Plant-derived insecticidal lectins and protease inhibitors

Detail about lectins and protease inhibitors have been discussed in earlier section. Some other insecticidal roles are summarized here. Besides insecticidal potential, GNA and ASAL also serve as a carrier protein for other insecticidal peptides and proteins to the haemolymph of lepidopteran larvae. It has been demonstrated by feeding GNA-allatostatin and GNA-SFI1 fusions to the tomato moth *Lacanobia oleracea* [133–135]. SFI1 is a neurotoxin isolated from the spider *Segestria florentina*. The individual toxin did not cause toxicity through oral delivery; however, the fusion proteins with GNA were toxic.

Lectins are reported to be insecticidal towards sap-sucking insects, where Bt-toxins are not effective. Transgenic tobacco expressing garlic (*Allium sativum*) leaf lectin showed substantial

control over peach potato aphids [136]. Fusion of galactose-binding domain of the non-toxic ricin B-chain with Cry1Ac provides additional binding domains, which increases interactions with the gut receptors in target insects. Transgenic rice and maize expressing the fusion protein show high toxicity in comparison to the Bt-toxin alone [137].

Protease inhibitors (PIs) expressing transgenic plants are not as effective as Bt and insecticidal lectin expressing plants. This is due to the adaptation in gut proteases in phytophagous insects. High genetic diversity in gut proteases and low potency of protease inhibitors is responsible for such adaptation. The combination of inhibitors (potato PI-II and carboxypeptidase) is not enough to avoid the compensatory adaptation [68]. However, inhibitors like barley trypsin inhibitor [65], equistatin from sea anemone [138], other cystatins [139, 140] or use of multiple inhibitors [141] or combination of inhibitors and lectins [142] might also be useful to provide resistance against insects in transgenic plants.

### 3.2.3. Multiple insecticidal proteins containing transgenic crop

Second-generation Bt transgenic cotton [Bollgard II (Cry1Ac + Cry 2Ab) and Widestrike (Cry1Ac + Cry1F)] are developed to increase the level of resistance against cotton bollworm [143, 144]. It has also been demonstrated that the expression of three insecticidal proteins (Cry1Ac, Cry2A and GNA) into Indica rice control three major pests, rice leaf folder (*Cnaphalocrocis medinalis*), yellow stem borer (*Scirpophaga incertulas*) and the brown plant hopper (*Nilaparvata lugens*) [145]. Cry proteins target the leaf folder and the stem borer, and GNA targets the plant hopper. Comparison of three different Bt-cotton lines (either single Cry1Ac or Cry2Ab, or both genes) for insect damage showed that the lines containing two Bt genes performed better [144]. Broccoli expressing both Cry1Ac and Cry1C exhibited increased resistance to diamondback moths and delayed the resistance development [146, 147]. Similarly, transgenic tobacco expressing Cry1Ac and cowpea trypsin inhibitor (CpT 1) delayed resistance development in *H. armigera* [148]. Recently, Bharathi et al. [149] pyramided two lectin genes ASAL and GNA and showed increased resistance against brown plant hopper, green leaf hopper and white backed plant hopper, as compared to their parental lines expressing single lectin. The performance of transgenic plant pyramided with genes has shown that the insecticidal functions of most of the toxins are non-overlapping and non-competitive.

### 3.2.4. Tissue-specific or regulated expression

Insecticidal proteins are usually expressed under constitutive promoter for higher accumulation of the proteins. Although the constitutive expression has some advantages, tissue-specific or inducible expression is desirable under certain circumstances. Insect attacks epidermal cells first and therefore the expression of insecticidal proteins under epidermal cell-specific promoters can be a useful strategy. For example, CER6 is an epidermal cell-specific promoter responsible for the expression of an enzyme for cuticular wax production [150]. Similarly, phloem-feeding insects can be targeted by using phloem-specific promoter like PP2 promoter of pumpkin [151], rice sucrose synthase Rss promoter [152] and root phloem-specific promoter AAP3 [153]. Tissue-specific expression of several insecticidal proteins has demonstrated as a good potential for insect control in several studies. Phloem-specific expression of ASAL under

promoter *Asu1* protects tobacco against aphid, *Myzus nicotianae* [154]. Transgenic chickpea expressing ASAL under *rolC* promoter showed effective control over *A. craccivora* [155] and transgenic Indian mustard (*Brassica juncea*) expressing ASAL under *Rss I* promoter showed resistance against aphid *Lipaphis erysimi* [136]. Researchers are also working on sap-sucking pest inducible phloem-specific promoters, which are not only insect-inducible but also insect-specific in nature [156]. Another strategy is temporal expression of insecticidal proteins as some insects infest a crop in a particular phase only. For example, pink bollworm (*Pectinophora gossypiella*) attacks and feeds on the cotton bolls only. At this stage, cotton plants are mature; the expression of Cry toxins goes down and becomes insufficient for effective control.

### 3.2.5. Strategies to over express secondary metabolites

Secondary metabolites synthesized by the plants participate in a number of physiological and biochemical processes. Our group demonstrated that the over-expression of pectin methylesterase of *Arabidopsis thaliana* and *Aspergillus niger* in transgenic tobacco plants enhances methanol production, which in turn provided resistance against sap-sucking as well as chewing insect pests [157]. Similarly, transgenic tobacco expressing *AtMYB-12* gene showed enhanced production of rutin in leaves and callus, which confers resistance against *H. armigera* and *S. litura* larvae [158, 159]. *WsSGTL1*, a sterol glycosyltransferases isolated from *Withania somnifera*, was expressed and functionally characterized in transgenic tobacco plants, which showed significant resistance towards *S. litura* [160]. Tobacco plants were transformed by a multigene transfer vector containing three coffee N-methyltransferases genes *CaMXT1*, *CaMXMT1* and *CaDXMT1* responsible for producing caffeine in transgenic plants which showed tolerance to *S. litura* [161]. Dixit et al [162] demonstrated the insect resistance by altering the amino acid composition in sap.

## 4. Conclusions and perspectives

Transgenic technology (especially Bt crops) has contributed significantly in increasing the crop production worldwide. The crops are protected from being damaged by insect pests. Certainly, this methodology provides an environmentally safe alternative for the synthetic pesticides. Further, it has also been proven to be useful in enhancing nutritional values of crops, improvement of stress tolerance and production of pharmaceutical proteins. Introduction of Bt cotton varieties in India has tremendously increased the yields of cotton and thereby profits to the farmers. Bt proteins are able to control the damage caused by Lepidopteran and Coleopteran insects, but not effective against sap-sucking Homopteran pests [8, 9]. Therefore, an unusual increase in the population of homopteran pests like whiteflies, aphids and leafhoppers on transgenic cotton has been reported [7]. Further, development of resistance in insects against toxins is also going to be a major point of concern, which might ultimately challenge the future of Bt crops. Some defense-related proteins like plant lectins, PIs and chitinases are reported to be toxic to various homopteran insect pests. However, several safety and societal concerns are raised from time to time. Further, there is non-availability of an effective and safe protein against several important and emerging insects, which need an *ab*

*initio* approach to resolve this issue. A promising and biosafe strategy to defeat the above problem can be: (a) exploration of the plant's own defense mechanisms and manipulation of their expressions or (b) by introducing a gene for insect control derived from other plants, especially derived from non-host plants, and (c) pyramiding of insecticidal proteins for the control of multiple insect pests. Exploration of RNAi mediated insect control by targeting high expressing and/or important vital genes can also be an effective approach (12-14, 163, 164). Besides this, in our country, we need a dedicated forum to popularize the use of genetically modified crops and convince the government as well as citizens at ethical issues.

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# Evaluation of TMV Lesion Formation and Timing of Signal Transduction during Induction of Systemic Acquired Resistance (SAR) in Tobacco with a Computer-Assisted Method

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Additional information is available at the end of the chapter

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## Abstract

*Nicotiana tabacum* L. cv. Xanthi nc plants were inoculated with tobacco mosaic virus (TMV) in order to develop a method for evaluation of lesion size and its distribution characteristics during the induction of systemic acquired resistance (SAR). All necrotic lesions were scored with an image analysis software and subjected to statistical analysis. The diminished lesion size and its right-skewed, non-normal distribution seem to be an important feature of SAR response. The results showed that the degree of induced resistance differs according to the position of the leaf on the plant's shoot. In order to detect the timing of signal transduction from TMV infected leaves to distant ones, the infected leaves were removed from the tobacco plants at different time intervals. When the infected leaves were removed after 4 days, the SAR was always induced on the distant leaves indicating complete signal transduction within 4 days.

**Keywords:** Lesion size and distribution, signal transduction, SAR, TMV, tobacco

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## 1. Introduction

Systemic acquired resistance (SAR) is a defence response that initiates immunity to a wide range of pathogens in distant uninfected leaves after a former localised necrotic infection of plants. It was described after tobacco mosaic virus (TMV) infection in tobacco by Ross [1]. In agricultural practice, SAR has been recognised as a strategy to control plant pathogens because of its evolutionary stability [2], long-lasting effectiveness [3] and putative transgenerational effect [4]. Not only pathogen infections but also a number of chemicals and biotic factors

including hormonal compounds (salicylic acid and methyl jasmonate), benzothiadiazole, Ningnanmycin (an antiviral agent against TMV) and insect eggs have been shown to induce SAR [5-9]. The signal transduction process and its timing are essential components of SAR induction that moves from induced to distant leaves. Recent studies indicated that the movement of SAR signal(s) is connected to plasmodesmata [10].

Induction of SAR is often validated by the increased expression of marker genes in distant tissues and/or by limited symptom expression/multiplication of the pathogen. However, the exact evaluation of symptom expression often has serious limitations. Visual assessment of leaf spots may lead to false analysis [11]. The number of developing TMV lesions is influenced by many factors (especially by the inoculation method and physiological state of plants) and therefore not well suitable as a single factor for characterization of the development of SAR [1,12].

In the present contribution we analysed local TMV lesion size formation and its distribution after SAR induction by a computer-assisted method. The method can detect nearly all lesions on tobacco leaves including the smallest ones (ca. 0.2 mm) and consequently is suitable for the exact determination and comparison of lesion size distribution of differentially treated plants. It is particularly important if a certain component is not normally distributed and mean of data is masking the fine differences. Sherwood [13] rejected normal distribution of fungal lesions (orchardgrass—*Stagonospora arenaria* interaction) in resistant genotypes, but not in susceptible ones. However, to the best of our knowledge, there is no systematic research on lesion size distribution of viral local necrotic infections.

Therefore, our tasks were as follows:

- a. To develop a reliable and well-adaptable method for semi-automated measurement of lesion size and its distribution using an appropriate statistical analysis (see Materials and methods)
- b. To evaluate and compare TMV lesion size distribution in control versus SAR plants
- c. To determine the timing of signal transduction process(es) by comparing lesion size distributions of distant leaves after removal of TMV-infected signal-inducing leaves at different time intervals

## 2. Materials and methods

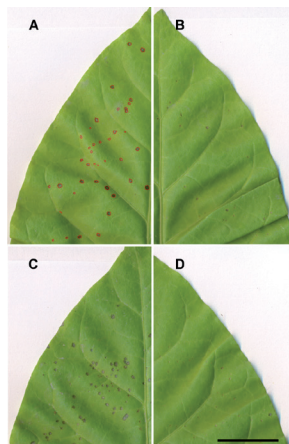
*Nicotiana tabacum* L. cv. Xanthi nc plants were grown in greenhouse and inoculated with TMV U1 strain at 6 leaf stage (fully expanded leaves) as we have described earlier [12]. The four bottom-most leaves of plants were inoculated for induction of systemic acquired resistance without any further treatments (treatment: SAR) [1]. A subset of these plants was further treated: the inoculated leaves were removed from the plants 2 and 4 days after inoculation (treatments: leaf removal, LR2+TMV and LR4+TMV, respectively). As a control, leaves were removed similarly from uninoculated plants (treatments LR2 and LR4). Seven days after inoculation, the two fully expanded distant leaves (the 5th and 6th leaf levels) were used for challenge inoculation and data analysis. Abrasive (carborundum, 50 mg/100ml) was added to

the inoculum. Mock inoculation was performed only with abrasive in 10 mM K-phosphate buffer (pH = 7.0).

Four days after challenge inoculation two or three detached leaves (of the same leaf level) per treatment, were directly scanned to obtain high resolution (300 dpi) digital images with a scanner (HP Scanjet G2k710). Data were collected from these images after threefold magnification on the computer screen. All dark, brownish black, round-shaped TMV lesions typical of this infection on tobacco plants were selected visually for analysis. The ImageJ 1.48v image analysis software [14] was used for lesion selection and size calculations. However, due to the low contrast of small TMV spots, lesion selection was done manually using a drawing tablet that outperforms the precision of a standard computer mouse. Lesion size was expressed as the mean of the major and minor axes of the best fitting ellipse having equal area to the lesion. Considering all lesions per leaf resulted in more accurate estimation of lesion size compared to former approaches [7,12,13]. Figure 1 demonstrates this process on TMV-infected tobacco leaves.

All calculations (lesion size distribution, mean and variance of lesion size) were carried out with R [15]. Shapiro-Wilk  $w$  test for normal distribution and density estimation of data were calculated with functions 'shapiro.test' and 'density', respectively with their default settings.

For comparison of sample means a multiple comparison procedure was used with the R package multcomp [16]. The method allows simultaneous comparisons while the family-wise error rate, used as the standard measure for false positive results in multiple testing, remains well controlled [17]. The method uses the HC3 covariance estimation [18,19]. Furthermore, this method tolerates unequal variances, non-normal distribution of data and unbalanced group sizes, which often occur in biological datasets.



**Figure 1.** Leaf necrosis on *Nicotiana tabacum* cv. Xanthi nc leaves after challenge inoculation. (A): control, (B): SAR, (C): the plants were not inoculated with TMV, but their leaves were removed after 4 days (LR4), (D): the four inoculated leaves were removed from the plants 4 days after inoculation (LR4+TMV). Bar: 3 cm. Red circles on (A) mark the processed lesions during image analysis.

3. Results and discussion

3.1. Effect of SAR induction on TMV lesion size and its distribution

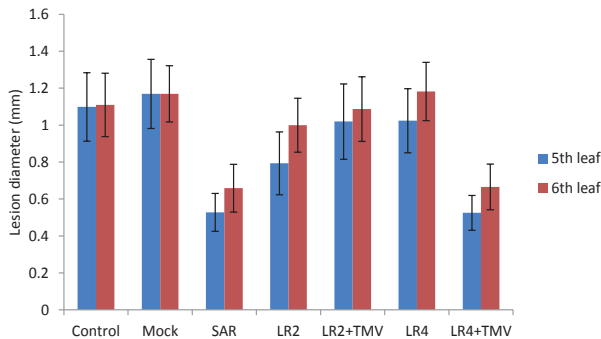
Data from Figs. 2, 3 and 4 are based on one representative experiment (Table 1) and serve as an example of three separate experiments. Mock inoculation with abrasive had no effect on lesion diameter and its distribution compared to control plants (Figs. 2, 3A, B and 4A, B). Induction of SAR was clearly manifested in differences of lesion development (Fig. 1) and in lesion size (Fig. 2) 4 days after challenge inoculation in all experiments. Although in control plants no significant differences were found between 5th and 6th leaf levels (Fig. 2), induction of SAR caused significant differences in degree of diminishing TMV lesion size between the 5th and 6th leaf levels. The effect of SAR induction often was more pronounced on the 5th leaf level than the 6th one (Fig. 2). Therefore, data for 5th and 6th leaves are presented separately in Figs. 2, 3 and 4. Generally, mean lesion diameter of leaves with SAR (0.528 and 0.659 mm for 5th and 6th leaf, respectively) was about half of the leaves from control plants (1.099 – 1.110 mm) (Fig. 2, Table 1).

Treatments	Leaf No.	n <sup>1</sup>	Lesion diameter (mm)			Normality test	
			Min	Max	Mean	Shapiro-Wilk's w	p
Control	5	343	0.25	2.01	1.099	0.9906	0.027
LR2	5	656	0.20	2.30	0.793	0.9396	< 0.001
LR2 + TMV	5	471	0.28	2.35	1.019	0.9747	< 0.001
LR4	5	428	0.29	2.27	1.024	0.9897	0.004
LR4 + TMV	5	187	0.22	1.32	0.525	0.8847	< 0.001
Mock	5	311	0.32	2.08	1.169	0.9929	0.150
SAR	5	226	0.23	1.31	0.528	0.8570	< 0.001
Control	6	132	0.43	1.80	1.110	0.9781	0.031
LR2	6	250	0.38	1.66	1.000	0.9835	0.005
LR2 + TMV	6	408	0.41	2.07	1.087	0.9851	< 0.001
LR4	6	145	0.46	1.96	1.182	0.9891	0.320
LR4 + TMV	6	145	0.29	1.35	0.666	0.9159	< 0.001
Mock	6	154	0.32	1.89	1.170	0.9889	0.266
SAR	6	103	0.25	1.55	0.659	0.9195	< 0.001

<sup>1</sup>For abbreviations and explanations of treatments see Fig. 2.

<sup>2</sup>Number of lesions.

**Table 1.** Test for normal distribution and lesion size data of TMV inoculated *Nicotiana tabacum* cv. Xanthi nc plants.

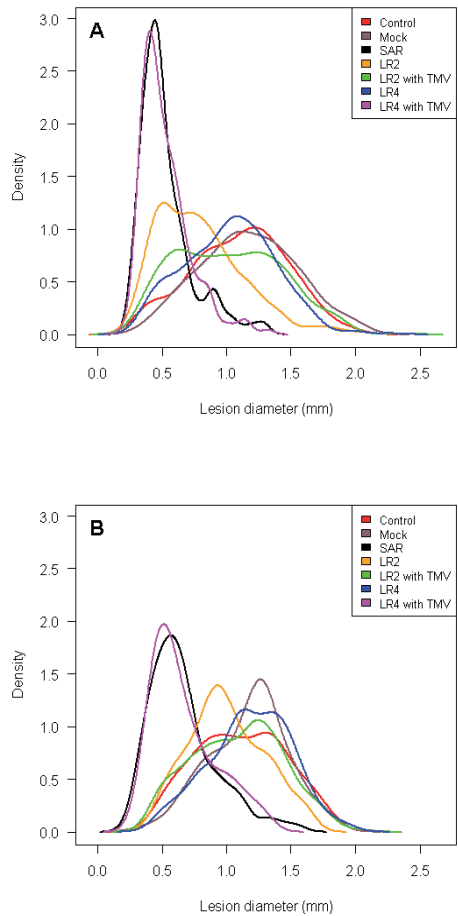


**Figure 2.** Mean lesion size (and standard deviation) after challenge inoculation with TMV on *Nicotiana tabacum* cv. Xanthi nc plants at leaf level 5 and 6. The four bottom-most leaves of plants were first inoculated with TMV and challenged on the 7th day. The four inoculated leaves were removed from the plants 2 and 4 days after inoculation (LR2+TMV and LR4+TMV, respectively). LR2 and LR4: the plants were not inoculated with TMV, but their leaves were removed at similar time intervals. Control: untreated plants; mock inoculated plants were treated with abrasive only; SAR: inoculated with TMV and challenged 7 days later without further treatments [1,12].

The distribution of TMV lesion size in most cases did not follow a normal distribution neither in control nor in leaves with SAR and other treatments as indicated by the results of Shapiro-Wilk  $w$  test (Table 1). Therefore, we used a statistical method suitable for comparison of non-normally distributed data. Comparison of multiple sample means under heteroscedasticity also showed highly significant differences ( $P < 0.001$ ) between control leaves and leaves with SAR both in the 5th and 6th leaf levels (Fig. 4A, B).

Supporting the above data, the distribution of lesion sizes in control leaves and in leaves with SAR was massively different both in 5th and 6th leaves (Fig. 3A, B). The lesion sizes in control plants showed a plateau-like distribution and covered a wide range from 0.25 to 2.01 mm and 0.43 to 1.80 mm on leaves 5 and 6, respectively (Table 1). The distribution of lesions in resistant leaves with SAR was completely different showing a peak at 0.3–0.8 mm range (about 70-80% of total number of lesions) and above 1.5 mm size, practically no lesions were detected (Fig. 3A, B, Table 1).

TMV causes local hypersensitive necrotic lesions in tobacco plants carrying *N* gene from *Nicotiana glutinosa* L. [20], accompanied by programmed cell death and development of symptoms within 2 days after inoculation. This resistant response is further strengthened during SAR induction as indicated by limited lesion size and a different type of lesion size distribution as compared to control plants. Similar to our results, a non-normal distribution of necrotic spots was reported in a resistant plant–fungus interaction [13]. This shift in lesion size distribution is probably due to biochemical responses that are manifested in more effective restriction of lesion development, multiplication/growth and/or movement of the pathogen in resistant genotypes. It has been reported recently that an antiviral agent against TMV can induce SAR in tobacco [9].



**Figure 3.** Kernel density estimation of TMV lesion size distribution on *Nicotiana tabacum* cv. Xanthi nc leaves at leaf level 5 (upper panel, A) and 6 (lower panel, B) after induction of systemic acquired resistance (SAR). For abbreviations and explanations of treatments see Fig. 2.

**3.2. Effect of sequential removal of inducing leaves on SAR development**

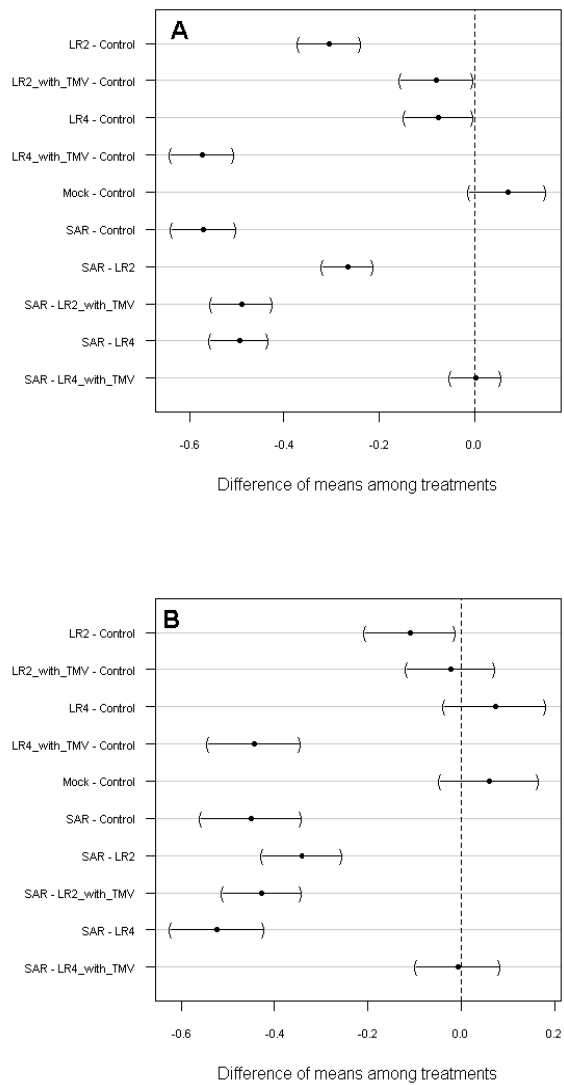
In order to detect the timing of signal transduction process from infected leaves to distant leaves, four bottom-most infected leaves were removed from tobacco plants at different time intervals, 2 (leaf removal, LR2) or 4 (LR4) days after TMV infection (Figs. 2, 3 and 4). The leaf removal without TMV infection after 4 days did not result in a significant shift in lesion



development (LR4, Figs. 1 and 2, Table 1). Moreover, distribution of lesion sizes showed a plateau-like picture, comparable to control leaves (Fig. 3). Simultaneous comparison of treatments also showed that LR4 treated plants did not significantly differ from control plants at least on the 6th leaf level ( $p = 0.3500$  with confidence interval:  $[-0.0353; 0.1805]$ , Fig. 4B). On leaf level 5 the effect was almost the same as on level 6 indicating a limited effect ( $p = 0.0346$  with confidence interval:  $[-0.146; -0.003]$ , Fig. 4A). On the contrary, removal of TMV-infected leaves after 4 days (LR4+TMV) mimicked the development of SAR in all characteristics in all three experiments. Lesion development was considerably, about 50% inhibited (Figs. 1, 2 and Table 1). The statistical analysis of data clearly showed highly significant differences between LR4+TMV and control plants ( $p < 0.001$  for both leaf levels) but no significant differences between LR4+TMV and SAR treatments at both leaf levels (Fig. 4A, B). Not surprisingly, the distribution of lesion development of LR4+TMV plants was nearly the same as in leaves with SAR showing a characteristic peak at about 0.5 mm of lesion diameter. (Fig. 3A, B). These results clearly indicate that a 4-day period after the inducing infection of lower 4 leaves is enough for complete signal transduction of SAR in distant leaves. Consequently, the movement of signal molecule(s) should be detectable before this time point. These results also indicate that lesion size distribution as a resistance marker is a suitable tool for prediction of signalling events. Similar experiments with removal of leaves after a 2-day interval (LR2 and LR2+TMV) showed less clear evidences. Leaf removal without TMV infection (LR2) considerably influenced lesion development in all experiments (Fig. 2, Table 1) and somewhat shifted distribution of lesion size at both leaf levels (Fig. 3A, B). LR2 plants showed significant differences as compared either to control or SAR treated plants (Fig. 4A, B). This fact could be related to a different mechanism as compared to SAR induction, for example differences in hormone balance of distant leaves during longer incubation period after leaf removal. In LR2+TMV plants, the development of SAR was not detected on the basis of lesion size and its distribution characteristics (Figs. 2, 3A, B). Family-wise comparison of data rather suggests that LR2+TMV plants did not significantly differ from control ones but differed from the SAR treatment ( $p = 0.0316$ , confidence interval:  $[-0.1543; -0.0044]$ ), Fig. 4A, B).

Altogether, these results indicate that the signal transduction starts probably only after visual appearance of local TMV symptoms (40–48 h post inoculation) and it is completed within the next 2 days. The identification of the exact timing of signal transduction from induced leaves is necessary for the further characterization of signal molecule(s) in phloem sap-enriched petiolar exudates.

In conclusion, we developed an easily applicable semi-automated method for the detection of the size of necrotic lesions and its distribution in tobacco leaves after TMV inoculation using appropriate statistical analysis. Decreased lesion size diameter and its characteristic non-normal, right-skewed distribution seem to be an accurate and important feature of the resistant response in distant leaves with SAR. Application of this method during SAR induction indicated that signal transduction is completed in distant leaves by the 4th day after inducing TMV inoculation. Further experiments are in progress to characterize the chemical nature of this signal.



**Figure 4.** Multiple comparisons of group means of selected treatments on leaf level 5 (A) and 6 (B). Dots represent the difference of the estimated means between treatments. Brackets flank the 95% confidence intervals. The difference is considered significant if the confidence interval does not contain the 0, represented by a vertical dashed line. Abbreviations and explanations are the same as on Fig. 2.

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# Abiotic Stress Alleviation with Brassinosteroids in Plant Roots

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Additional information is available at the end of the chapter

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## Abstract

This chapter covers the advances in establishment and optimization of brassinosteroids (BRs) in the alleviation of abiotic stresses such as water, salinity, temperature, and heavy metals in plant system, especially roots. Plant roots regulate their developmental and physiological processes in response to various internal and external stimuli. Studies are in progress to improve plant root adaptations to stress factors. BRs are a group of steroidal hormones that play important roles in a wide range of developmental phenomena, and recently they became an alleviation agent for stress tolerance in plants. This review is expected to provide a resource for researchers interested in abiotic stress alleviation with BRs.

**Keywords:** Water stress, salt stress, temperature stress, heavy metal stress

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## 1. Introduction

Abiotic stress responses in plants occur at various organ levels among which the root-specific processes are of particular importance. Under normal growth condition, root absorbs water and nutrients from the soil and supplies them throughout the plant body, thereby playing pivotal roles in maintaining cellular homeostasis. However, this balanced system is altered during the stress period when roots are forced to adopt several structural and functional modifications. Examples of these modifications include molecular, cellular, and phenotypic changes such as alteration of metabolism and membrane characteristics, hardening of cell wall, and reduction of root length [1, 2]. The root system has the crucial role of extracting nutrients and water through a complex interplay with soil biogeochemical properties and of maintaining these functions under a wide range of stress scenarios to ensure plant survival and reproduction [3].

Water stress is characterized by a reduction of water content and leaf water potential, closure of stomata, and decreased growth. Severe water stress may result in the arrest of photosynthesis, disturbance of metabolism, and finally the death of plant [4]. This water loss causes a loss of turgor pressure that may be accompanied by a decrease in cell volume depending on the hardness of the cell wall [5]. The cells of the root must activate processes to limit water loss and mitigate its harmful effects.

Salinity also affects plant growth, activity of major cytosolic enzymes by disturbing intracellular potassium homeostasis, causing oxidative stress and programmed cell death, reducing nutrient uptake, genetic and epigenetic effects, metabolic toxicity, inhibition of photosynthesis, decreasing CO<sub>2</sub> assimilation, and reducing root respiration [6, 7, 8, 9]. Salt stress affects the root in all developmental zones. Cell division decreases in the meristematic zone and cell expansion attenuates in the elongation zone, resulting in reduced overall growth [10]. Cells also expand radially in the elongation zone [11], and root hair outgrowth suppresses in the differentiation zone [12]. Salt stress additionally results in agravitropic growth [13] as well as reduced lateral root number under high-salt conditions and enhanced lateral root number under moderate-salt conditions [14, 15]. Salt stress develops from excessive concentrations of salt, especially sodium chloride (NaCl) in soil. Root is the primary organ of exposure and hence responds rapidly [16]. Salt stress is known to increase Na<sup>+</sup>/K<sup>+</sup> ratio in the root that leads to cell dehydration and ion imbalance [17, 18, 19].

High temperature increases the permeability of plasma membrane [20], and also reduces water availability [21]. Moreover, low temperature (chilling and frost stress) is also a major limiting factor for productivity of plant indigenous to tropical and subtropical climates [22]. Chilling stress has a direct impact on the photosynthetic apparatus, essentially by disrupting the thylakoid electron transport, carbon reduction cycle, and stomatal control of CO<sub>2</sub> supply, together with an increased accumulation of sugars, peroxidation of lipids, and disturbance of water balance [23].

Heavy metal contamination in soil could result in inhibition of plant growth and yield reduction and even poses a great threat to human health via food chain [24]. Among heavy metals, Cadmium (Cd) in particular causes increasingly international concern [25]. Cd-contaminated soil results in considerable accumulation of Cd in edible parts of crops, and then it enters the food chain through the translocation and accumulation by plants [26, 27]. Another metal, chromium (Cr III or VI), is not required by plants for their normal plant metabolic activities. On the contrary, excess of Cr (III or VI) in agricultural soils causes oxidative stress for many crops. Reactive oxygen species (ROS), like hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (OH<sup>•</sup>), and superoxide radical (O<sub>2</sub><sup>•-</sup>) generated under Cr-stress, are highly reactive and cause oxidative damages to DNA, RNA, proteins, and pigments [28, 29]. Nickel (Ni) is one of the most abundant heavy metal contaminants of the environment due to its release from mining and smelting practices. It is classified as an essential element for plant growth [30]. However, at higher concentrations, nickel is an important environmental pollutant. Ni<sup>2+</sup> ions bind to proteins and lipids such as specific subsequences of histones [31] and induce oxidative damage. Copper (Cu) is also an essential micronutrient for most biological organisms. It is a cofactor for a large array of proteins involved in diverse physiological processes, such as

photosynthesis, electron transport chain, respiration, cell wall metabolism, and hormone signaling [32, 33]. Cu has emerged as a major environmental pollutant in the past few decades because of its excessive use in manufacturing and agricultural industries [34]. Zinc (Zn) is one of the other essential microelement, the second most abundant transition metal, and plays roles in many metabolic reactions in plants [35, 36]. However, high concentrations of Zn are toxic, induce structural disorders, and cause functional impairment in plants. At organism level, Zn stress reduces rooting capacity, stunted growth, chlorosis, and at cellular level alters mitotic activity [37, 38].

The key to find out abiotic stress tolerance resides in understanding the plant's capacity to accelerate/maintain or repress growth. Most plant hormones play a role in development and have been implicated in abiotic stress responses. One of these hormones, BRs, are a group of steroidal hormones that play significant roles in a wide range of developmental phenomena including cell division and cell elongation in stems and roots, photo-morphogenesis, reproductive development, leaf senescence, and also in stress responses [39]. Mitchell et al. [40] discovered BRs which were later extracted from the pollen of *Brassica napus* by Grove et al. [41]. To date, more than 70 BR-related phytosteroids have been identified in plants [42].

BRs increase adaptation to various abiotic stresses such as light [43, 44], low or high temperature [45], drought [46, 47, 48], salt stress [9], and heavy metal stress [49, 50]. BRs may be applied/supplied to plants at different stages of their life cycle such as meiosis stage [51], anthesis stage [52], and root application [9, 53].

In this chapter, the potential role of BRs in alleviating the adverse effects of water, salt, low/high temperature stresses, and heavy metals on plants, especially roots, were discussed.

## 2. Water stress

Water shortage is predicted as one of the most important environmental problem for the 21st century that limits crop production [54]. Although drought stress inhibites the plant-water relations, exogenous application of BRs maintains tissue–water status [55] by stimulating the proton pumping [56], activating nucleic acid and protein synthesis [57] and regulation of genes expressions [58]. It has been shown that 24-epibrassinolide (24-epiBL)-treated *Arabidopsis* and *B. napus* seedlings had a higher survival rate when subjected to drought [59], and in another study BR-treated sorghum (*Sorghum vulgare*) showed increased germination and seedling growth under osmotic stress [60].

Root nodulation is a fundamental developmental event in leguminous crops, and is sensitive to water shortage [61, 62, 63]. As endogenous hormones play an important role in the organogenesis and initial growth of nodules in roots, attempts have been made to increase root nodulation by growth regulator treatments [64, 65]. The potential of BRs in the improvement of root nodulation and yield have been reported in groundnut [66]. Upreti and Murti [67] also studied the effects of two BRs, epibrassinolide (EBL) and homobrassinolide (HBL), on root nodulation and yield in *Phaseolus vulgaris* L. cv. Arka Suvidha under water stress. They

concluded that water stress negatively influenced nodulated root, but BRs increased tolerance to water stress and EBL was relatively more effective than HBL.

Several researchers have found that increased proline levels can protect plants from water stress. BR treatment increased the contents of proline and protein under water stress [68]. Zhang et al. [69] also indicated that BR treatment promoted the accumulation of osmoprotectants, such as soluble sugars and proline. It may be due to the fact that BRs activated the enzymes of proline biosynthesis, which caused an additive effect on the proline content [70].

Drought stress causes increment in H<sub>2</sub>O<sub>2</sub> due to decrease in antioxidative enzyme activities [71]. Plants have improved various defense mechanisms to respond and adapt to water stress [72]. Vardhini et al. [73] studied with sorghum seedlings grown under PEG-imposed water stress and investigated the effects of HBL and 24-epiBL on the activities of four oxidizing enzymes: superoxide dismutase (SOD), glutathione reductase (GR), IAA oxidase, and polyphenol oxidase (PPO). They found that supplementation of both the BRs resulted in enhanced SOD and GR but lowered IAA oxidase and PPO. Li and Feng [68] also reported that treatment of brassinolide significantly increased peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) activities of seedlings under normal water and mild water stress. Therefore, increment in enzyme activities provided tolerance of *Xanthoceras sorbifolia* seedlings to drought stress. It has been found that BRs can induce the expression of some antioxidant genes and enhance the activities of antioxidant enzymes such as SOD, POD, CAT, and APX [74, 75].

3. Salt stress

Salinity stress is one of the most serious abiotic stress factors. It causes morphological, biochemical, cytogenetic, and molecular changes in plants [9, 76, 77, 78]. Root lengths, shoot lengths, and root numbers decrease in plants exposed to salt stress [7]. Moreover, salinity also induces oxidative stress in plants due to production ROS [79, 80]. These ROS are produced in the cell and interacted with a number of vital cellular molecules and metabolites, thereby leading to a number of destructive processes causing cellular damage [81].

BRs reduce impacts of salt stress on ROS, gene expression, mitotic index, nutrient uptake, and growth [9, 82, 83–88]. There are lots of studies to analyse alleviation of salt stress by using BRs. In these studies, different parameters have been investigated to understand the mechanism of BRs on salt stress (Table 1).

References	[9]	[84]	[85]	[86]	[87]	[88]
					Increased	
Effects on					Cu/Zn-SOD,	
gene expression	---	---	---	---	APX, CAT,	---
					GR and OsBRI	
					expressions	



					but reduced <i>Fe-SOD</i> and <i>Mn-SOD</i> , <i>OsDWF4</i> and <i>SalT</i>	
<b>Effects on protein content</b>	Increased	---	Increased	---	Increased	---
<b>Effects on enzyme activities</b>	Increased SOD and CAT activities	Increased CAT, GR, POX and SOD activities	Increased POX and SOD activities	Increased SOD and POD activities	Shown varying results depending on 24-epiBL concentration for SOD, APX, CAT, GR	Increased CAT, POX and SOD activities
<b>Effects on growth and/or cell division</b>	Increased	Increased	Increased	Increased	Increased	Increased
<b>Methods of salt and BRs applications</b>	Seeds were grown under both 150–250 mM salt concentrations and 0.5 and 1 $\mu$ M HBR at 48 h and 72 h.	Plants received 100 mM NaCl as well as 0.01 $\mu$ M of HBL during 18 days after sowing	25, 50, 100, and 150 mM NaCl were applied and then sprayed twice with 0.05 ppm brassinolide during 25 days. At 45 days from sowing, the plants were collected	Seedlings were exposed to 90 mM NaCl with 0, 0.025, 0.05, 0.10, and 0.20 mg dm <sup>-3</sup> 24-epiBL for 10 days	Seeds were soaked for 8 h in different concentrations of 24-epiBL ( $10^{-11}$ , $10^{-9}$ and $10^{-7}$ M). After 24-epiBL application, The seeds were sown in autoclaved sand moistened with different concentrations of NaCl (0, 75, 100, 125 mM) during 12 days	The 15-day-old plants were exposed to 100 mM NaCl and they were subsequently treated by exogenous 24-epiBL ( $10^{-8}$ M). The plants were harvested after 30 days of growth
<b>Plant Species</b>	<i>Hordeum vulgare</i> L.	<i>Vigna radiata</i> L.	<i>Vigna sinensis</i> L.	<i>Solanum melongena</i> L.	<i>Oryza sativa</i> L. var. Pusa Basmati-1 cv. indica	<i>Cucumis sativus</i> L.

**Table 1.** Effects of BRs on plants subjected to salt stress Dashes indicate that there are no results in study.

## 4. Temperature stress

### 4.1. High temperature

In general, a transient elevation in temperature (usually 10–15°C above environment) causes heat shock or heat stress [89]. High-temperature effects can be seen at the biochemical and molecular level in plant organs (especially leaves). Heat stress induces decrease in duration of developmental phases, leading to fewer organs, smaller organs, reduce light perception over the shortened life cycle, and finally play an important role in losing the product [90, 91, 92].

High-temperature stress often induces the overproduction of ROS [93] which can cause membrane lipid peroxidation, protein denaturation, and nucleic acid damage [94, 95]. Many studies have demonstrated that ROS scavenging mechanisms play an important role in protecting plants from high-temperature stress [96, 97]. BRs applications decrease ROS levels and increase antioxidant enzyme activities to provide thermotolerance to elevated temperatures [98].

### 4.2. Low temperature

Chilling and frost stresses affect growth, development, survival, and crop productivity in plants [99, 100, 101]. However, BRs treatments enhance seedling tolerance to chilling stress [101] and increase the height, root length, root biomass, and total biomass of rice under low-temperature conditions [102, 103]. In another study, Krishna [104] reported the same results in maize. They postulated that treatments with BRs promoted growth recovery of maize seedlings following chilling treatment (0–3°C).

Chilling stress increases the proline, betaine, soluble protein, soluble sugar contents of plants [79, 105]. Studies showed that BRs treatment enhanced proline content and therefore increased plant chilling resistance and cell membrane stability [99, 100, 106, 107].

Chilling stress could trigger the production of antioxidant enzymes in plants to prevent the chilling injury [108]. In the previous investigations, it was reported that treatment with BRs further increased the activities of antioxidant enzymes under chilling stress as well [99, 100, 107, 109]. The enhanced activities of the antioxidative enzymes as a result of BRs applications may occur with increasing *de novo* synthesis or activation of the enzymes, which is mediated through transcription and/or translation of specific genes to gain tolerance [57].

## 5. Heavy metal stresses

### 5.1. Cd stress

Cd toxicity has emerged as one of the major agricultural problems in many soils around the world [110]. It has been shown to interfere with the uptake, transport, and utilization of essential nutrients and water, change enzyme activities, cause symptoms (chlorosis, necrosis), decrease in fresh and dry mass of root and shoot and also their lengths [110, 111, 112].

There are lots of studies to investigate the effects of BRs on Cd stress in plant species [110, 113, 114]. In these studies, results showed that BRs change different parameters such as germination, plant dry biomass, protein content, and antioxidant enzyme activities (Table 2). It is proposed that the changes induced by BRs are mediated through the repression and/or de-repression of specific genes [58]. Microarray experiments evaluating gene expression changes in *Arabidopsis* roots and shoots under Cd stress were performed [115]. Moreover, studies showed that gene expression in response to Cd mimics a BR increase, and Cd exposure most probably induces an activation of the BR signaling pathway in *Arabidopsis* [116].

## 5.2. Cr stress

Cr (III or VI) is not required by plants for their normal plant metabolic activities [117]. The entry of Cr into a plant system occurs through roots via using the specialized uptake systems of essential metal ions required for normal plant metabolism [118]. On the contrary, excess of Cr (III or VI) in agricultural soils causes oxidative stress to many crops. Reduced seed germination, disturbed nutrient balance, wilting, and plasmolysis in root cells and thus effects on root growth of plants have been documented in plants under Cr stress [118, 119].

Choudhary et al. [120] reported that EBL treatment improved seedlings growth under Cr (VI) stress. Ability of EBL to increase seedling growth under this metal stress could be attributed to the capacity of BRs to regulate cell elongation and divisional activities, by enhancing the activity of cell wall loosening enzymes (xyloglucan transferase/hydrolase, XTH) [121]. Studies also indicated that increment in antioxidant activities as a result of BRs application (Table 2) provide plant tolerance to grow under Cr stress.

## 5.3. Ni stress

The heavy metals that affect (either positively or negatively) plants include Fe, Cu, Zn, Mn, Co, Ni, Pb, Cd, and Cr, but out of them, nickel has recently been defined as an essential micronutrient, because of its involvement in urease activity in legumes [122]. Excess Ni causes different problems. These symptoms include the inhibition in root elongation, photosynthesis and respiration, and interveinal chlorosis [123]. Moreover, the toxic concentration of Ni also inhibits enzyme activities and protein metabolism [124]. This metal also accelerates the activities of antioxidative enzymes [125, 126].

BRs effect on Ni stress in plants has been studied to understand the relationship between BRs and this stress (Table 2). One of these studies was carried out by Yusuf et al. [49]. They showed that seed germination and seedling growth were significantly reduced by Ni treatment, but HBL treatment enhanced germination percentage as well as shoot and root lengths in Ni-stressed seedlings. BRs confer tolerance against heavy metals either by reducing their uptake or by stimulating the antioxidative enzymes in *B. juncea* [127, 128]. The exogenous application of BRs in nickel-stressed *R. sativus* L., and *Triticum aestivum* L. plants enhanced the pool of antioxidant enzyme activity, thus alleviating the toxic effects of this stress [129].

5.4. Cu stress

Among the pollutants of agricultural soils, Cu has become increasingly hazardous due to its involvement in fungicides, fertilizers, and pesticides [130]. In addition, Cu present in excess has been known to decrease root biomass and alter plant metabolism [131, 132]. Sharma and Bhardwaj [127] demonstrated decrease in growth parameters of *Brassica juncea* grown under Cu stress. The reduction in growth parameters due to the Cu stress occurred as a result of decreasing mitotic activity and cell elongation [133, 134]. Moreover, Chen et al. [130] suggested a different opinion. They concluded that Cu-induced inhibition in root growth of rice seedlings was due to the stiffening of the cell wall. Moreover, excess of Cu ion leads to the generation of harmful ROS via the formation of free radicals [135].

Effects of exogenous application of BRs were studied on *Raphanus sativus* seedlings under Cu stress. It was found that 24-epiBL promoted the shoot and root growth by overcoming the Cu toxicity [136]. The growth-promoting effects of BRs on seedlings under Cu stress may be linked to the general ability of BRs to promote cell elongation and cell cycle progression [137, 138] as well as the stimulation of genes encoding xyloglucanases and expansins [139]. BRs applications also increase antioxidant enzyme activities [140, 141]. Increasing all parameters as a result of BRs application improves plant tolerance against Cu stress, and finally plant development (Table 2).

5.5. Zn stress

Zn is an essential microelement, the second most abundant transition metal after iron (Fe), and has a role in many metabolic reactions in plants [35, 36]. However, high concentrations of Zn are toxic, induce structural disorders, and cause functional problems in plants. At organism level, Zn stress causes reduced rooting capacity, growth, and at cellular level alters mitotic activity [37, 38]. It induces oxidative stress by promoting ROS production as a result of indirect consequence of Zn toxicity [142].

Application of BRs on plants alleviates Zn stress via increasing protein content and antioxidant enzyme activities (Table 2). Çağ et al. [143] reported that EBL application effectively enhanced the protein content in *Brassica oleraceae* cotyledons. Sharma et al. [144] also reported that pre-sowing treatments of HBL lowered the uptake of metal and enhanced the activities of antioxidant enzymes and protein concentration of *B. juncea* seedlings under Zn stress. Moreover, Ramakrishna and Rao [145] also reported that the application of 24-epiBL significantly alleviated the Zn-induced oxidative stress.

References	[114]	[146]	[30]	[147]	[141]	[145]
Effects on gene expression	---	---	---	---	---	---
Effects on protein content	---	Increased	Decreased protein content	---	Showed varying results	---

Effects on enzyme activities	Increased CAT, POX and SOD activities	Decreased CAT level	Decreased SOD but increased POD activities	Increased SOD, CAT and POX activities	Shown varying results	Increased
Effects on growth and/or cell division	Alleviated	Improved	Improved	Improved	Increased	Increased
Methods of metal and BRs applications	Soil amended with 50 $\mu\text{M}$ Cd and foliage sprayed with $10^{-8}$ M HBL at 20 days after sowing. The plants were sampled at 30 days after sowing	Seeds were treated with eEBL ( $10^{-9}$ M) and 1.2 mM Cr(VI) ( $\text{K}_2\text{CrO}_4$ ) solution at 7 days	Seeds were soaked for 8 h in different concentrations of 24-epiBL ( $0$ , $10^{-7}$ , $10^{-8}$ and $10^{-11}$ M). Then, seeds were grown under different ( $0$ , $0.5$ , $1.0$ , $1.5$ , and $2.0$ mM) Ni concentrations	Seeds were grown in different levels of $\text{Cu}^{2+}$ ( $50$ or $100$ mg $\text{kg}^{-1}$ of soil. At 15 and 20 days stage, $10^{-5}$ mM HBL was applied. At 45 days, plants were collected	Seeds were grown under both $30$ $\mu\text{M}$ – $40$ $\mu\text{M}$ Cu and $2$ $\mu\text{M}$ HBR at 48 h and 72 h	EBR ( $0.5$ , $1$ , and $2$ $\mu\text{M}$ ) and $5$ mM of Zn were applied to seeds. Seven day old seedlings were collected
Plant Species	<i>Triticum aestivum</i> L.	<i>Raphanus sativus</i> L.	<i>B. juncea</i> L.	<i>Vigna radiata</i> L.	<i>Helianthus annuus</i> L.	<i>Raphanus sativus</i> L.
	Cd Stress	Cr Stress	Ni Stress	Cu Stress		Zn Stress

**Table 2.** Effects of BRs on plants subjected to Cd, Cr, Ni, Cu and Zn stresses. Dashes indicate that there are no results in study

## 6. Conclusion

Roots are very important plant organs whose architecture is determined by endogenous and environmental conditions to adjust water and nutrient uptake from soil [148, 149]. BRs, one of the plant hormones, have both positive and negative effects on root growth related to hormone concentrations [150]. Experimental condition is one of the most important factors for analysing BRs effects on root development. The procedures using BRs to alleviate abiotic systems generally are easy, time saving, and one of the most reliable systems [53]. Therefore, BRs open up new approaches for plant tolerance against hazardous environmental conditions [151]. Morphological, biochemical, and molecular analyses have been performed to analyse the effects of BRs. However, detailed analyses should be performed to investigate the relationship between abiotic stresses and BRs, especially gene expression studies will provide knowledge about interaction at molecular level in plants [152]. We tried to cite as many papers as possible. Yet we apologize to authors whose works are gone unmentioned in this chapter.

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## **Role of Phytochelatins in Redox Caused Stress in Plants and Animals**

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Additional information is available at the end of the chapter

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### **Abstract**

Varied environmental compartments (such as soil and water) potentially contaminated with different metals/metalloids can impact the health of both plants and animals/humans. Trace amounts of Cu, Mn, Mo, Ni and Zn are beneficial for higher plants, whereas, Cr, Cu, Co, Mn, Mo, Se, V and Zn are known as the micronutrient metal/metalloids for animals/humans. However, elevated levels of the metals/metalloids can cause severe toxic consequences in both plants and animals/humans. Common in plants and animals/humans, phytochelatins (PCs), the principal non-protein, S-rich, thiolate peptides, protect (through different mechanisms) cellular functions and metal/metalloid homeostasis by performing their chelation and/or detoxification. With the major aim of broadening the current knowledge on the subject, this chapter (a) overviews PCs' role and modulation separately in metal/metalloid-exposed plants and animals/humans; (b) discusses major methods for determination of PCs and bioassays for enzymes involved in PC synthesis; (c) evaluates the connection of PCs with bionanoparticles; and finally (d) highlights so far unexplored aspects in the present context.

**Keywords:** Phytochelatin, metal, glutathione, stress

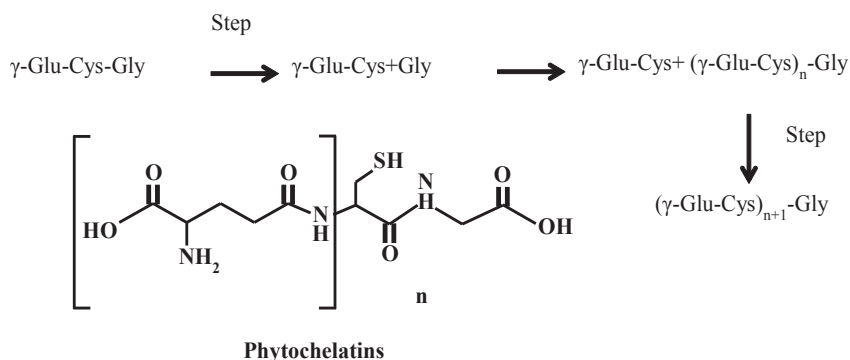
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### **1. Introduction**

Anthropogenic activities have caused the release of a wide range of hazardous metals/metalloids (hereafter termed as 'metal/s') into the environment. In particular, increasing emissions of metals such as Cd, Hg and As into the environment pose an acute problem for all organisms. Metals, unlike organic contaminants, are not degradable and remain persistent in soils [1–3]. Once taken up, these metals can bring severe toxic consequences in cells due to their chemical similarity to replace the metals necessary for cellular functions. Nevertheless,

metals at toxic levels have the capability to interact with several vital cellular biomolecules such as nuclear proteins and DNA, leading to excessive augmentation of reactive oxygen species (ROS) [4–6]. In addition, these metals generate ROS which in turn can cause neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals [7, 8]. Notably, higher plants, algae, certain yeasts and animals are equipped with a repertoire of mechanisms to counteract metal toxicity. The key elements of these are chelation of metals by forming phytochelatins (PCs) and related cysteine-rich polypeptides [9–11]. PCs are produced from glutamine, cysteine and glycine and the process is catalysed by PC synthases known as  $\gamma$ -glutamylcysteine ( $\gamma$ -Glu-Cys) dipeptidyl transpeptidases [12, 11]. PCs have been identified in a wide variety of plant species, microorganisms and invertebrates. They are structurally related to glutathione (GSH) and were presumed to be the products of a biosynthetic pathway. Numerous physiological, biochemical and genetic studies have confirmed that GSH is the substrate for PC biosynthesis [13, 14]. The general structure of PCs is  $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ , with increasing repetitions of the dipeptide Glu-Cys, where  $n$  can range from 2 to 11 but is typically no more than 5 [15]. Except glycine, other amino acid residues can be found on the C-terminal end of  $(\gamma\text{-Glu-Cys})_n$  peptides. In Figure 1, we show the general structure of PC and the major steps involved in its synthesis from GSH through PC synthase in response to high concentrations of toxic metals. Originally thought to be plant-specific, PC and PC synthases have now been reported in a few fungal taxa, such as the yeast *Schizosaccharomyces* sp. and the mycorrhizal ascomycete *Tuber melanosporum* [16, 17] and invertebrates belonging to the nematodes, annelids or plathyhelminths [18, 19, 4, 1, 20, 17, 21–24].

In the light of recent literature, the PCs' role and modulation are overviewed separately in metal-exposed plants and animals/humans and major methods for the determination of PCs and the bioassays for enzymes involved in PC synthesis are discussed hereunder. Additionally, connection of PCs with bionanoparticles is evaluated, and finally, major aspects so far unexplored in the present context are briefly highlighted.



**Figure 1.** General structure of phytochelatins (PCs) and the major steps involved in its synthesis from glutathione (GSH) through a PC synthase in response to high concentrations of toxic metals.

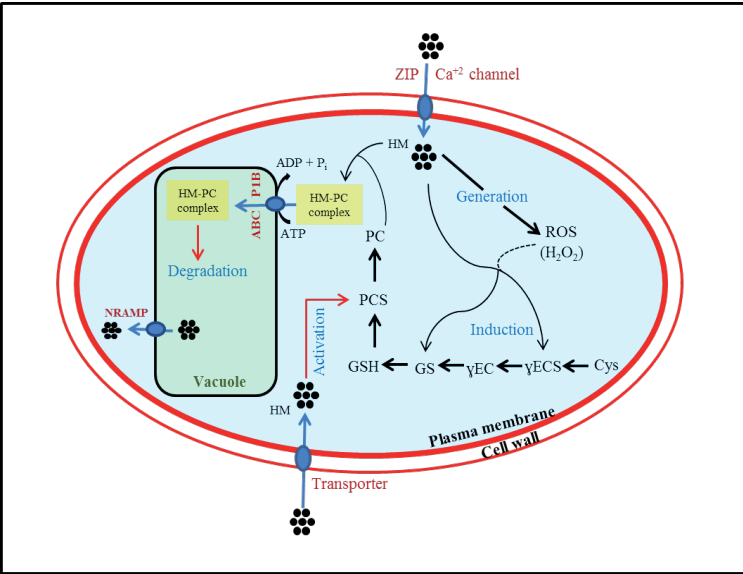
## 2. Phytochelatins in metal/metalloid-exposed plants

Contamination by metals can be considered as one of the most critical threats to soil and water resources as well as to human health [25, 26]. In fact, the contamination of soils with toxic metals has often resulted from human activities, especially those related to accelerated rate of industrialization, intensive agriculture and extensive mining. Metal belongs to group of non-biodegradable, persistent inorganic chemical having cytotoxic, genotoxic and mutagenic effects on humans or animals and plants through influencing and tainting food chains, soil, irrigation or potable water and aquifers [27, 28, 6]. Chelation and sequestration of metals by particular ligands are the major mechanisms employed by plants to deal with metal stress. The two best-characterized metal-binding ligands in plant cells are the PC and metallothioneins (MTs) [29–33, 6, 34].

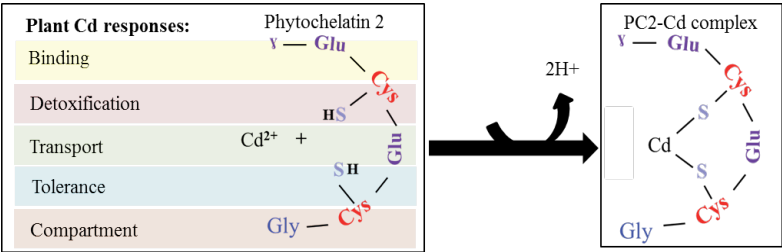
Figure 2 shows the scheme of metal-detoxification by PCs in a plant cell. PC, which has a higher affinity for Cd, is formed by the polymerization of 2–11  $\gamma$ -EC moieties via PC synthase. Several studies confirm that in plants, both GSH and PC synthesis are increased after exposure to Cd and other metals [12, 35–41]. In Figure 3, we show both general functions of the PC and a model of complex between  $\text{Cd}^{+2}$  ion and one molecule of PC.

Gonzalez-Mendoza et al. showed that PC synthase gene (in coordination with the expression of metallothionein gene) is present in *Avicennia germinans* leaves, and that their expression increases in response to metal exposure, which supports the hypothesis that PC synthase and metallothionein are part of the metal-tolerance mechanisms in this species. In addition, these authors found that *A. germinans* has the ability to express both genes (*AvMT2* and *AvPCS*) as a coordinated response mechanism to avoid the toxic effects caused by non-essential metals. However, for essential metals such as  $\text{Cu}^{2+}$ , the results showed that *AvPCS* was the most active gene involved in the regulation of this metal in the leaves [42]. Recent study showed that *Lunularia cruciata* compartmentalizes  $\text{Cd}^{+2}$  in the vacuoles of the photosynthetic parenchyma by means of a PC-mediated detoxification strategy, and possesses a PC synthase that is activated by Cd and homeostatic concentrations of Fe(II) and Zn. *Arabidopsis thaliana* PC synthase displays a higher and broader response to several metals (such as Cd, Fe(II), Zn, Cu, Hg, Pb, As(III)) than *L. cruciata* PC synthase [35].

Naturally hyperaccumulating plants do not overproduce PCs as a part of their mechanism against toxic metals. This appears to be an inducible rather than a constitutive mechanism, observed especially in metal non-tolerant plants [43]. Some reports have argued against the roles of PC in some metal-tolerant plants based on the effects of buthionine-S-sulphoximine and PCs/metal concentrations [44]. Several studies on plants overexpressing  $\gamma$ -glutamyl-cysteine synthetase or transgenic plants expressing bacterial  $\gamma$ -glutamyl-cysteine synthetase evaluated its effect on metal tolerance based on the assumption that higher levels of GSH and PCs will lead to more efficient metal sequestration [45]. *Bacopa monnieri*, a wetland macrophyte, is well known for its accumulation potential of metals and metal tolerance and thus is suitable for phytoremediation. Aquatic plants respond to metal stress by increasing the production of PC as well as other antioxidants. The accumulation potential of *B. monnieri* for various metals warrants its evaluation for metal tolerance and detoxification mechanism and for its suitability



**Figure 2.** The scheme of heavy metal (HM) detoxification by phytochelatin (PC) in a plant cell. HM activates phytochelatin synthase (PCS) and the HM-PC complexes are established. These complexes are consequently transported through tonoplast to vacuole by ATP-binding-cassette and P1B-ATPase transporter (ABC-PIB). HM is chelated in the cytosol by ligands such as PC. Induction of PC synthesis by HM and a large flux of GSH is further achieved by increased activity of the GSH metabolic enzymes,  $\gamma$ -ECS and GS. It is possible that the enzyme activation is not directed through effects of HM but due to H<sub>2</sub>O<sub>2</sub> produced as a result of HM-presence. Transport of HM through the plasma membrane (ZIP). Vacuolar transport of HM (NRAMP: natural resistance associated macrophage protein). Heavy metals are shown as black dots. Figure adapted and modified from [26].



**Figure 3.** General functions of phytochelatin (PCs) and the model of complex between cadmium ( $\text{Cd}^{2+}$ ) ion and one molecule of PC2. Cys, cysteine; Glu, glutamic acid; Gly, glycine; S, sulphur.

in phytoremediation [38]. In a study on *Arabidopsis thaliana* showed that Cd is immediately scavenged by thiols in root cells, in particular PC, at the expense of GSH. At the same time, a redox signal is suggested to be generated by a decreased GSH pool in combination with an

altered GSH:GSSG ratio in order to increase the antioxidant capacity [46]. Overexpression of PCs synthetase in *Arabidopsis* led to 20–100 times more biomass on 250 and 300  $\mu\text{M}$  arsenate than in the wild type. Also, the accumulation of thiol-peptides was 10 times higher after the exposure to Cd and arsenic, compared to the wild type. Gamma-glutamyl cysteine, which is a substrate for PC synthesis, increased rapidly after arsenate or Cd-exposure. Overexpression of PC synthase gene can be useful for phytoremediation [47]. Additionally, legumes are also capable of synthesizing homo-PCs in response to metal stress [45]. Citrus plants were also reported to synthesize PC in response to metal intoxication [48]. In wheat (*Triticum aestivum*), PC–metal complexes have been reported to accumulate in the vacuole. Retention of Cd in the root cell vacuoles might influence the symplastic radial Cd transport to the xylem and further transport to the shoot, resulting in genotypic differences in grain Cd accumulation [49].

### 3. Phytochelatins in metal/metalloid-exposed animals

As mentioned also above, PC proteins have been broadly described and characterized in plants, yeasts, algae, fungi and bacteria [22]. However, PC synthase genes are also present in animal species from several phyla. PC synthesis appears not to be transcriptionally regulated in animals [50]. Nevertheless, originally thought to be found only in plants and yeast, PC synthase genes have since been found in species that span almost the whole animal tree of life. Notably, PC synthase genes are found in species from several other metazoan phyla, including Annelida, Cnidaria, Echinodermata, Chordata and Mollusca (both Gastropoda and Bivalvia classes) [51, 52].

Several phyla of the Metazoa contain one or more species harbouring PC synthase homologous sequences: the Cnidaria (*Hydra magnipapillata*), the Chordata (*Molgula tectiformis*, as well as the model chordate *Ciona intestinalis*), the Echinodermata (*Strongylocentrotus purpuratus*), the Annelida (*Lumbricus rubellus*) and the Platyhelminthes (*Schistosoma japonicum* and *Schistosoma mansoni*) [53, 51]. Biochemical studies have also shown that these PC synthase genes are functional. The *Caenorhabditis elegans* PC synthase produces PC when it is expressed in an appropriate host, and knocking out the gene increases the sensitivity of *C. elegans* to Cd [54]. Several studies have since measured PC by direct biochemical analysis of *C. elegans* tissue extracts, and found that Cd exposure did indeed increase PC levels in *C. elegans*. PC2, PC3 and PC4 have all been found, with PC2 in the highest concentration [55, 20, 56]. Therefore, these studies concluded that PCs production can play a major role in protecting *C. elegans* against Cd toxicity. PC2 and PC3 were increased in autochthonous *Lumbricus rubellus* populations sampled from contaminated sites [50]. The yeast (for example, *S. pombe*) possesses an ATP-binding cassette (ABC) transporter, Hmt1, which was originally thought to play a possible role in translocation of PCs–metal complexes to the vacuole. However, while knocking out the *C. elegans* HMT-1 (CeHMT-1) increases the sensitivity to Cd; the increase is greater than could be explained by a lack of PC synthase alone [57]. It is important to say that MTs are another widely established metal-binding ligand and a key metal detoxification system in animals. Additionally, MTs have many other important biological functions

as well. Nevertheless, little is known about how MTs and PCs may complement each other for dealing with toxic metals [50].

The activation and function of PC synthase in animals came into light from studies on the nematode *C. elegans* [58], the flatworm *Schistosoma mansoni* [19, 59, 21], and Cionidae *Ciona intestinalis* [60]. The occurrence of PC synthase in animals suggests the occurrence, in these organisms, of a stress oxidative and metal detoxification system based on a class of molecules which was considered as the privilege of plants. The PC synthase gene has a wide phylogenetic distribution and can be found in species that cover almost all of the animal tree of life. But even though some members of particular taxonomic groups may contain PC synthase genes, there are also many species without these genes. Ron Elran et al. reported the regulation of GSH cycle genes in *Nematostella vectensis*, and an interesting finding was that PC synthase 1, which synthesizes the non-ribosomal formation of metal-binding PC, was upregulated after Hg and Cu treatments [15]. Phylogenetic analyses supported the hypothesis that PC synthase evolved independently in plants, cyanobacteria and green algae. Among the sequenced metazoan genomes, only a few contain a PC synthase gene. However, the reason for the scattered distribution of these genes remains unclear, considering that metazoans with PC synthase genes in their genomes do not share any physiological, behavioural or ecological features [60]. Just how (and if) PC in invertebrates complement the function of MTs remains to be elucidated, and the temporal, spatial and metal specificity of the two systems are still unknown [6].

## 4. Methods for the assays of phytochelatins and phytochelatin synthase enzyme

### 4.1. Determination of phytochelatins

We briefly discuss herein different methods for the detection and quantification of PC. Additionally, we are giving an overview of the methods used for determination of PC, comprising a broad range of electrochemical as well as spectrometric methods, which have been optimized and even hyphenated with different separation methods to detect PC. Recently, Wood et al. showed the analytical methodology for quantification of PC and their metal(loid) complexes [61]. The classical approach to the analysis of PC is reversed phase HPLC with post-column derivatization of the sulphhydryl groups and spectrophotometric detection, but the detection is not specific to PC. The use of an analytical technique is able to detect compounds, specifically mass spectrometry. Independent studies showed a sensitive method for determining PCs by HPLC with fluorescence detection [62, 63]. A simple sensitive method for the identification, sequencing and quantitative determination of PCs in plants by electrospray tandem mass spectrometry (ESI MS-MS) was showed for different studies [64, 65]. Other study showed the combination of three processes for identification PC: (1) easy sample preparation including thiol reduction, (2) rapid and high-resolution separation using ultra-performance liquid chromatography (UPLC) and (3) specific and sensitive ESI-MS/MS detection using multi-reaction mode (MRM) transitions in alga's extract [66].



Nevertheless, in vitro formed Cd-PC2 complexes were characterized using ion exchange chromatography (IEC), flow injection analysis/high-performance liquid chromatography with CoulArray or Coulochem electrochemical detector and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [67, 68]. Zitka et al. optimized high-performance liquid chromatography coupled with electrochemical detector for determination of PC2 [69]. Many studies showed the determination of cysteine, reduced and oxidized glutathione and PC in different species of plants using high-performance liquid chromatography with electrochemical detection [70, 71].

#### 4.2. Bioassays for phytochelatin synthase activity

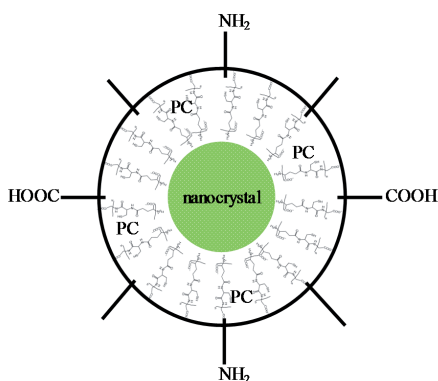
The methods for identification and quantification of PC synthase are multidisciplinary, among themselves, comprising a broad range of molecular biology, electrochemical and spectrometric methods. HPLC coupled with electrochemical detector has been suggested as a new tool for the determination of PC synthase activity. The optimized procedure was subsequently used for studying PC synthase activity in the tobacco BY-2 cells treated with different concentrations of Cd(II) ions and the results were in good agreement with Nakazawa et al. [72]. Other study in animals showed that HPLC-LC system coupled to a single quadrupole LC-MS equipped with ESI was a sensitive method for PC synthase activity [22]. A highly sensitive assay for PC synthase activity was devised, where, the dequenching of Cu(I)-bathocuproinedisulphonate complexes was used in the detection system of a reversed-phase high-performance liquid chromatography. The present assay method is a sensitive tool that can be used to investigate this issue and would allow for the determination of PC synthase activity using 10–100-fold less protein [73]. Electrochemical methods such as differential pulse voltammetry and high-performance liquid chromatography with electrochemical detection were used for determination of Pt(IV) content, GSH levels, PC synthase activity in maize (*Zea mays*) and pea (*Pisum sativum*) plants treated with various doses of Pt(IV) [74].

Other methods required for the identification and characterization of PC synthase are, for example, the novel technology of molecular biology. Xu et al. showed a study that represents the first transcriptome-based analysis of miRNAs and their targets responsive to Cd stress in radish (*Raphanus sativus*) roots. Furthermore, a few target transcripts including PC synthase 1 (PCS1), iron transporter protein and ABC transporter protein were involved in plant response to Cd stress [75]. In 2009, Amaro et al. reported the identification and characterization of a cDNA encoding a PC synthase homologous sequence from the ciliated protozoan *T. thermophila*, the first to be described in ciliates. A quantitative real-time PCR (qRT-PCR) expression analysis of PC synthase has been carried out under different metal stress conditions. Several experimental evidences suggest that this enzyme is biosynthetically inactive in PC formation, which makes it the first pseudo-PC synthase to be described in eukaryotes [76].

### 5. Phytochelatins in connection with bionanoparticles

The connection of nanoparticles and PC has two faces: on one hand, the biosynthesis of nanoparticles and on the other hand, the protection of stress caused by the damage of any

harmful nanoparticles. An *in vitro* study showed the enzyme-mediated synthesis of CdS nanocrystals by immobilized PC synthase, which converts GSH into the metal-binding peptide PC. Formation of CdS nanocrystals were observed upon the addition of CdCl<sub>2</sub> and Na<sub>2</sub>S with PC as the capping agent [77]. This study is expected to help in designing a rational enzymatic strategy for the synthesis of nanoparticles of different chemical compositions, shapes and sizes. Also, an enzymatic synthesis route to peptide-capped gold nanoparticles was developed. Gold nanoparticles were synthesized using alpha-NADPH-dependent sulphite reductase and PC *in vitro* [78]. In Figure 4, we show the general structure of nanocrystal with cross-linked, PC-like coating (Figure modified from [79]). The microbiological production of inorganic nanoparticles is an interesting and promising alternative to the known physical and chemical production methods. Extensive studies revealed the potential of bacteria, actinomycetes, algae, yeasts and fungi for biosynthesis of nanoparticles [80]. Few studies have discussed the possible synthesis of nanoparticles by algae. Particularly, *Phaeodactylum tricornutum* exposed to Cd, forms Cd-PC complexes, where sulphide ions (S<sup>2-</sup>) can be incorporated to stabilize PC-coated CdS nanocrystallites [81, 82]. Metal is immobilized by an intracellular detoxification mechanism. Krumov et al. showed that Cd is associated to a protein fraction between 25 and 67 kDa which correspond to the theoretical molecular weight of CdS nanoparticles of 35 kDa coated with PC by size exclusion chromatography [83]. However, contingent to their types and concentrations, any nanoparticles can pose a risk to human health and to the environment [84]. Zinc oxide nanoparticles (ZnONPs) are used in large quantities by the cosmetic, food and textile industries. The harmful effects of ZnONPs are driven by their physicochemical properties and the resulting physical damage caused by the aggregation and agglomeration of nanoparticles. PC synthase may confer protection against ZnONPs-induced toxicity in *Caenorhabditis elegans* [24]. Effect of magnetic nanoparticles on tobacco BY-2 cell suspension culture showed induced PC biosynthesis. These trends were observable for almost all monitored PCs: PC2, PC3 and PC5 [85].



**Figure 4.** Nanocrystal with crosslinked, phytochelatin (PC)-like coating, an effective strategy to make QDs as small with a crosslinked peptide sheath by mimicking PC-coated heavy metal nanoclusters. Figure adapted and modified from [79].

## 6. Conclusions

The concept of phytoremediation of contaminated soils has been increasingly supported by research in recent years. The identification of PC synthase genes from plants and other organisms is a significant breakthrough that will lead to a better understanding of the regulation of a critical step in PC biosynthesis. Many studies showed the mechanisms of chelation of metals–PC in plants in recent years. Chelation and sequestration of metals by particular ligands are also mechanisms used by plants to deal with metal stress. The two best-characterized metal-binding ligands in plant cells are the PCs and MTs. While the role played by PC synthase enzymes and PCs in animals still remains to be fully explored, there is increasing evidence that PC synthase genes are likely to be found in many important animal groups and that PCs may well turn out to be important players in metal ion detoxification in many of these species. It will be of interest in the future to see whether different animal species coordinate PC and MT responses to potentially toxic elements and if this is different for different metal ions.

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# Signalling Crosstalk of Plant Defence Responses to Xylem-invading Pathogens

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Additional information is available at the end of the chapter

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## Abstract

Xylem is a plant vascular tissue that transports water and dissolved minerals from the roots to the rest of the plant. It consists of specialized water-conducting tracheary elements, supporting fibre cells and storage parenchyma cells. Certain plant pathogenic fungi, oomycetes and bacteria have evolved strategies to invade xylem vessels and cause highly destructive vascular wilt diseases that affect the crop production and forest ecosystems worldwide. In this chapter, we consider the molecular mechanisms of root-specific defence responses against vascular wilt pathogens, with an emphasis on the most important and well-studied fungal (*Verticillium* spp. and *Fusarium oxysporum*) and bacterial (*Xanthomonas* spp. and *Ralstonia solanacearum*) pathogens. In particular, we present the current understanding of plant immune responses, from invasion perception to signal transduction and termination. Furthermore, we address the role of specific transcription factors involved in plant immunity and their regulatory network. We also highlight the crucial roles of phytohormones as signalling molecules in local and systemic defence responses. Finally, we summarize the current knowledge of plant defence responses to xylem-invading pathogens to devise new strategies and methods for controlling these destructive plant pathogens.

**Keywords:** Vascular wilt pathogens, effectors, plant innate immunity, signal transduction, biotic stress

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## 1. Introduction

The disease triangle concept, introduced in the 1960s by George McNew to predict plant disease outcomes, shows the complex interactions among the environment, the host and the infectious (or abiotic) agent [1]. Plants, continuously challenged by numerous abiotic stresses, potential pests and pathogens, have evolved efficient strategies to perceive and respond to

such threats. Plants lack specialized immune cells and their survival relies upon a highly sophisticated innate immune system, in which each plant cell responds autonomously [2–5]. The first line of defence is a basal resistance response called pattern-triggered immunity (PTI). It is induced by recognition of exogenous microbe or pathogen-associated molecular patterns (MAMPs or PAMPs) or endogenous molecules released on pathogen perception or pathogen-induced cell damage (damage-associated molecular patterns, DAMPs) via pattern-recognition receptors (PRRs) in the plasma membrane [4]. Successful pathogens overcome PTI by secreting effectors, hydrolytic enzymes or toxins, which suppress or interfere with host defence molecules [6]. In an evolutionary arms race, plants have evolved a robust defence response network termed effector-triggered immunity (ETI) to intercept pathogen effectors through intracellular receptors, such as nucleotide-binding site/leucine-rich repeat (NLR) proteins [5,7,8]. An intricate network of signalling pathways transduces these incoming signals into a diverse array of immune responses activating reactive oxygen species (ROS) generation, MAP kinases,  $\text{Ca}^{2+}$  signalling, the production of phytohormones and extensive transcriptional reprogramming [9].

In the past, comprehensive research has been dedicated to understanding plant physiological and molecular responses to individual abiotic and biotic stresses under controlled laboratory conditions. Recent studies of plant responses to concurrent abiotic and biotic stress conditions [10–16] have demonstrated that plants perceive and respond to combined stresses in a specific and unique manner. Moreover, the underlying signalling pathways are carefully modulated [14,17,18] and coordinated to ensure that plant growth and fitness are not significantly retarded [19].

Vascular wilt pathogens are soil-borne bacteria, fungi and oomycetes that employ various infection strategies to invade plant roots at different infection sites [20]. They subsequently advance inter- or intracellularly through the root cortex and enter the xylem vessels, where they proliferate and spread passively with xylem sap to aerial plant parts [21]. The characteristic wilt symptoms develop as a consequence of obstructed transportation of water and minerals, either due to the physical blockage of vessels by the pathogen or indirectly due to the activation of plant physical defence responses (e.g. formation of tyloses, accumulation of pectin-rich gels and gums) that confine the further spread of the pathogen [22]. In addition to wilting, other disease symptoms include vein clearing, leaf epinasty, chlorosis, vascular browning, stunting, necrosis and eventually plant death [21–24].

Primarily due to the specific lifestyle of vascular wilt pathogens, relatively little is known about their interactions with host plants and root-specific defence responses on molecular and biochemical levels compared to foliar pathogens. This chapter, therefore, summarizes the currently available molecular, cellular and systems biology data gathered from studies of signalling networks in model plants and crops challenged by bacterial or fungal pathogens and applies this general knowledge to advance understanding of vascular wilt pathogenesis and implement all these findings into the design of new strategies for the protection of crops and forest ecosystems.

## 2. Plant immune signalling initiation

Several factors contribute to the complex regulatory mechanisms in the initiation of plant immune signalling: (i) as sessile organisms, plants need to respond promptly to danger signals, (ii) each plant cell reacts autonomously to different stimuli, but the response needs to be integrated at a higher organizational level to ensure the plant's survival and (iii) immune reactions are energy- and resource-demanding processes requiring the proper timing and amplitude of response [4]. Typically, immune responses occur on recognition of conserved microbe-, pathogen- or damage-associated molecular patterns or after perception of effector molecules that are species-, race-, or strain-specific and contribute to pathogen virulence [25]. However, not all microbial elicitors conform to the common distinction between PAMPs and effectors, and so Thomma et al. [26] proposed that plant immunity should be considered as a continuum, instead of a two-branched system composed of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). An alternative perspective of plant innate immunity as a system that evolves to detect invasion has recently been extended into a so-called invasion model [27]. In this model, invasion patterns (IPs), externally encoded or modified-self ligands that signify invasion, are perceived by plant invasion pattern receptors (IPRs) and induce IP-triggered responses that do not result in immunity by default.

### 2.1. Plant PRRs convey danger signals to the intracellular immune signalling pathways

Recently emerging structural biology data on plant–pathogen interactions, [28] together with data obtained primarily from genetic and biochemical studies on the leaves of model plants and crops, have revealed that fine-tuning and coordination of immune responses are achieved within large protein complexes at the plasma membrane, where plant PRRs reside [4]. Plant PRRs are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) [4,6,25,29]. RLKs are modular proteins comprised of an extracellular domain involved in recognition of MAMPs/DAMPs, a single-pass transmembrane domain and a cytosolic serine/threonine kinase domain that transmits a signal to downstream signalling components. RLPs have a similar architecture, but their short cytoplasmic tail lacks kinase activity. RLPs, therefore, probably form heteromeric complexes with RLKs or other cytosolic kinases to relay downstream signalling. Extracellular domains of PRRs contain various motifs involved in recognition and binding of ligands. Leucine-rich repeat (LRR) motifs are widespread and serve as a scaffold for protein- or peptide–protein interactions [30]. Well-characterized examples of LRR–RLKs are *Arabidopsis* FLS2 (flagellin-sensitive 2) and EFR (elongation factor Tu receptor), which bind flagellin fragment flg22 and EF-Tu peptide elf18, respectively [31,32]. Lysine motifs (LysMs), lectin and epidermal growth factor (EGF)-like domains are found in PRRs that recognize carbohydrate moieties, such as fungal chitin [33,34] or bacterial peptidoglycans [35].

#### 2.1.1. Perception of chitin

The chitin-responsive PRR system has been thoroughly investigated in both dicots and monocots. The perception of chitin in monocot plants is best described in rice (*Oryza sativa*). Chitin elicitor binding protein (OsCEBiP) is a receptor-like protein that specifically binds chitin

oligomers [36]. In the absence of chitin, OsCEBiP exists as a homodimer [37]. On binding of chitin octamer, OsCEBiP associates with receptor-like kinase OsCERK1 and forms heterodimers [37,38]. This interaction activates the OsCERK1 kinase domain to become phosphorylated. Subsequently, active OsCERK1 phosphorylates a guanine nucleotide exchange factor OsRacGEF, which activates a Rho-type small GTPase OsRac [39]. OsRac acts as a molecular switch in many plant signalling pathways and, among other things, regulates the production of ROS by the NADPH oxidase OsRbohB [40].

The model dicot plant *Arabidopsis thaliana* harbours chitin elicitor receptor kinase 1/LysM-containing receptor-like kinase1 (CERK1/LYK1), which perceives chitin (a polymer of N-acetyl-D-glucosamine, NAG) through its LysM motif [33]. Although the AtCERK1 extracellular domain contains three tandem LysMs, only LysM2 binds NAG<sub>5</sub> [41]. This interaction, however, fails to trigger immune responses. Downstream signalling has been observed only on binding of chitin octamer, which acts as a bivalent ligand and induces CERK1 dimerization [41]. Another LysM-containing cell surface receptor, AtLYK5, has recently been proposed as the primary chitin receptor, due to a significantly higher binding affinity for NAG<sub>8</sub> compared to AtCERK1 [42]. AtLYK5 exists as a homodimer in the absence of chitin. Binding of chitin to AtLYK5 homodimer promotes the association of AtLYK5 with AtCERK1. This leads to dimerization of AtCERK1 and activation of its kinase domain. The chitin signal is then transduced downstream to mitogen-activated protein kinases MPK3 and MPK6 [33].

### 2.1.2. Perception of flagellin

Extensive research of the model plant *Arabidopsis thaliana* has elucidated molecular mechanisms triggered in response to recognition of bacterial flagellin by evolutionary conserved LRR RLK flagellin-sensitive 2 (FLS2) [31]. The extracellular domain of FLS2 contains 28 LRR and binds the 22-amino acid long flagellin epitope flg22 [43]. Immediately after, FLS2 associates with co-receptor brassinosteroid insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1)/somatic embryogenesis receptor-like kinase 3 (SERK3) to form a heterodimer [44–46]. BAK1 is a key regulatory LRR RLK coordinating growth–defence trade-offs [47], since it is required for early defence responses in PTI [48] but also implicated in brassinosteroid hormone signalling [49]. BAK1 phosphorylates receptor-like cytoplasmic kinase *Botrytis*-induced kinase 1 (BIK1), which interacts and forms a complex with both BAK1 and FLS2 [50]. BIK1 is subsequently auto-phosphorylated at tyrosine and serine/threonine residues [51]. Activated BIK1 contributes to flg22-triggered calcium influx from apoplast [52] and phosphorylates NADPH oxidase RbohD involved in reactive oxygen species (ROS) production [53].

### 2.1.3. Perception of peptidoglycan

Peptidoglycan (PGN), a polymer of N-acetylglucosamine and N-acetylmuramic acid branched with a short peptide, is an essential component of a bacterial cell wall and is another well-studied MAMP. In *Arabidopsis*, the PGN perception system is comprised of two GPI-anchored LysM domain RLPs, LYM1 and LYM3, which bind PGN, and a transmembrane RLK AtCERK1, which probably relays the PGN signal downstream [35]. Rice has a similar PGN detection

system, involving LysM RLK OsCERK1 [54] and two LysM-containing proteins, OsLYP4 and OsLYP6, which not only bind PGN but also associate with chitin oligomers [55].

#### 2.1.4. Perception of DAMPs

Some PRRs respond to damage-associated molecular patterns (DAMPs), endogenous molecules such as cell wall fragments or peptides released on pathogen attack or various abiotic stresses [25,56]. Partial degradation of pectic polysaccharide homogalacturonan (HGA) by pathogen or plant polygalacturonases produces oligogalacturonides (OGs), oligomers of  $\alpha$ -1,4-linked galacturonic acid [57,58]. In *Arabidopsis*, OGs are perceived by wall-associated kinase 1 (WAK1) Ser/Thr RLK kinase with an extracytoplasmic domain that contains several EGF-like repeats [59]. The signal is then relayed to the MAP kinase signalling pathway, where AtMPK3 and AtMPK6 become phosphorylated and induce expression of several defence genes [60]. Additional OGs-triggered defence responses include activation of NADPH oxidase AtRbohD involved in the generation of ROS, production of NO and deposition of callose in the plant cell walls [57,58].

The best studied peptides acting as DAMPs belong to the plant elicitor peptides (Peps) family. They are processed from precursor PROPEPs [61,62]. AtPep1, a 23 amino acid peptide released from the C-terminal of PROPEP1, was the first peptide elicitor isolated from *Arabidopsis* [61], but similar peptides were also later confirmed in other plants [63]. AtPep1 is recognized by two LRR RLK, PEPR1 and its paralog PEPR2 [64,65]. However, signalling is initiated only in complex with co-receptor LRR RLK BAK1 [66]. The active receptor complex consequently induces the expression of MAP kinase 3 (MPK3), WRKY transcription factors and defence-related genes such as *PR-1* and *PDF1.2* (encoding defensin) [63]. Moreover, the cytosolic kinase domain of PEPR1 has guanylyl cyclase activity, which generates cGMP from GTP [67]. An increased local concentration of cGMP has been proposed to open cyclic nucleotide-gated channels (CNGC2) in the plasma membrane and activate cytosolic  $\text{Ca}^{2+}$  signalling [67,68].

#### 2.2. Intracellular immune receptors NLRs detect pathogen effectors

In addition to PRRs, plants have evolved a second class of immune receptors that intercept effectors in different parts of the cell [8]. These intracellular receptors, so called NLRs, are characterized as multi-domain proteins that have a conserved central nucleotide-binding (NB) domain and variable C-terminal leucine-rich repeats (LRR) domain [28]. In terms of their distinct N-terminal domains, NLRs are broadly divided into two groups: TNLs that harbour a Toll-interleukin 1 receptor (TIR) domain and CNLs that contain a coiled-coil (CC) domain [69–71]. NLRs belong to signal transduction ATPases with numerous domains (STAND) that operate as molecular switches cycling between an inactive closed ADP-bound state and active open state with bound GTP [72–74]. In the resting state, N-terminal TIR or CC and C-terminal LRR domains sterically inhibit the NB domain from ADP–ATP exchange. On pathogen recognition, a series of conformational changes occur that expose the NB domain, promote ADP–ATP exchange and initiate signal transduction [8]. Effector recognition by NLRs often, but not always, leads to a form of programmed cell death termed as a hypersensitive response [75]. NLRs are, therefore, under precise control by accessory proteins. NLRs interact with conserved Hsp90-Sgt1-RAR1 protein complexes for proper folding, accumulation and



regulation [76,77]. Moreover, Sgt1 interacts with the suppressor of *rps4-RLD* (SRFR1) negatively to regulate NLRs accumulation and prevent autoimmune activation [78].

Various strategies of effector recognition by NLRs exist and are represented in different models. In the gene-for-gene model, originally described by Flor in 1971, NLRs can recognize effectors directly (e.g. *Pita-AvrPita* [79], *Pto-AvrPto* [80] and many others) acting like receptor and ligand pairs that initiate a cascade of reactions leading to resistance [3]. When considering pathogen effector diversity, it is more likely that a single NLR recognizes multiple effectors from diverse pathogens in the presence of other host proteins. This hypothesis is explained by the guard model, in which the NLR protein is assigned the role of a sentinel that is activated indirectly by detecting an effector-modified host protein and induces a defence response [81,82]. An example of such mechanism is given by *Arabidopsis* CNL receptors, RPM1 and RPS2, which constantly monitor host protein RIN4 (a negative regulator of basal resistance) for interference with *Pseudomonas syringae* effectors AvrB, AvrRpm1 and AvrRpt2 [83]. Another indirect effector recognition strategy is proposed in the decoy model [84]. As guarded effector targets are evolutionarily unstable, it is likely that the targeted host gene has duplicated and evolved into decoy proteins. These serve as bait to trap effectors that target structurally related proteins involved in basal defence. For example, in the inactive state, *Arabidopsis* CNL RPS5 interacts with its N-terminal CC domain with protein kinase PBS1, which has no function in basal resistance [85]. Cleavage of PBS1 by *Ps. syringae* effector AvrPphB activates RPS5 [86]. However, several PBS1-like kinases (including Botrytis-induced kinase 1 (BIK1)) are also cleaved by AvrPphB [87]. PBS1, therefore, acts as a decoy that prevents cleavage of BIK1, which is an important component of PRR signalling [53] and the key AvrPphB target. An integrated decoy model has recently been proposed [5]. In this model, the effector-targeted plant protein is duplicated and fused to one member of the NLR pair to act as bait that, on effector binding, triggers defence signalling by the second NLR.

Activated NLRs trigger a variety of immune responses, from the generation of ROS, elevation of intracellular  $\text{Ca}^{2+}$ , activation of MAPK cascades, transcriptional reprogramming to production of phytohormones [8]. Although effector-triggered responses are qualitatively similar to immune responses elicited by MAMPs/DAMPs, there are quantitative differences in the strength and duration of pathways, which result in different resistance responses and signalling networks [17].

### 2.3. Signal transduction cascades

Perception of MAMPs/DAMPs by their cognate receptors triggers an array of immune responses, comprising changes in intracellular calcium levels [ $\text{Ca}^{2+}$ ]<sub>i</sub>, membrane potential depolarization, extracellular alkalization, production of ROS, NO and phosphatidic acid, activation of kinases (mitogen-activated protein kinases (MAPKs) or  $\text{Ca}^{2+}$ -dependent protein kinases (CDPKs)), transcriptional reprogramming and changes in plant hormone concentrations (e.g. ethylene, salicylic and jasmonic acid) [88,89].

#### 2.3.1. Calcium and ROS signalling interconnection

$\text{Ca}^{2+}$  is a ubiquitous second messenger released in response to various stresses and developmental processes. In *Arabidopsis*, various MAMPs/DAMPs induce distinct and sustained



elevations of intracellular calcium concentration ( $[Ca^{2+}]_i$ ), which differ in the lag phase and amplitude of response [89,90]. Moreover, changes in  $[Ca^{2+}]_i$  are organ-specific and correlate with the expression patterns of the corresponding MAMP/DAMP receptors. Chitin octamer and Pep1 induce similar responses in seedling shoots and roots, while roots are insensitive to elf18 and show only a minor response to flg22 [90]. Furthermore,  $Ca^{2+}$  fluxes are generated from different sources; flg22/FLS2 signalling involves the release of  $Ca^{2+}$  from intracellular stores (e.g. endoplasmic reticulum and/or tonoplast) and inositol phosphate signalling, whereas Pep/PEPR signalling requires an influx of  $Ca^{2+}$  from the apoplast [68]. The identity of plant  $Ca^{2+}$  channels and pumps involved in the generation of  $Ca^{2+}$  signals is largely unknown, although some candidates (e.g. ionotropic glutamate receptor (iGluR)-like channels, cyclic nucleotide gated channels (CNGCs) and annexins in plasma membrane and two-pore-channel 1 (TPC1) in the tonoplast membrane) have been investigated [88,91]. Elevated  $[Ca^{2+}]_i$  is detected by  $Ca^{2+}$  sensor proteins such as calmodulins (CaMs), calcium-dependent protein kinases (CDPKs), calcineurin B-like (CBL) proteins and CBL-interacting protein kinases (CIPKs) [88,91]. CaMs are highly conserved eukaryotic proteins that bind free  $Ca^{2+}$  with four EF-hand motifs and regulate the function of their interacting proteins, such as CaM-binding transcription factors [88]. CDPKs are unique proteins acting as sensors and decoders of  $Ca^{2+}$  signals and are suited for rapid responses to stimuli. Binding of  $Ca^{2+}$  via four EF-hand domain motifs in the C-terminus activates the CDPKs' N-terminal kinase domain and promotes transmission of a  $Ca^{2+}$  signal by phosphorylating different target proteins [91]. The functional specificity of CDPKs is achieved by targeting distinct membrane subdomains and involves specific lipid modifications (e.g. N-terminal myristoylation, S-acylation) [91]. In contrast to CDPKs, CBLs are  $Ca^{2+}$  sensors without enzymatic activity. They bind  $Ca^{2+}$  with four EF hands and then associate with CIPKs through the NAF motif in the kinase C-terminal regulatory domain. This interaction liberates kinase from auto-inhibition and enables conversion of the  $Ca^{2+}$  signal into phosphorylation events [91]. Like CDPKs, CBLs have different lipid modifications (e.g. N-terminal myristoylation, S-acylation) that determine their localization and, consequently, the site of action of CBL–CIPK complexes [91].

Rapid production of reactive oxygen species (ROS) in response to MAMPs/DAMPs depends primarily on respiratory burst oxidase homologues (RBOHs) [92,93]. These NADPH oxidases are integral membrane proteins that generate superoxide anions ( $O_2^-$ ), which are rapidly converted into hydrogen peroxide ( $H_2O_2$ ). RBOHs have cytosolic FAD- and NADPH-binding domains in the C-terminal region, six membrane-spanning domains and a cytosolic N-terminal extension that harbours two EF-hand motifs and multiple phosphorylation sites [94]. Recent studies in *Arabidopsis* have revealed different regulation mechanisms of RBOHD and RBOHF-dependent ROS production. RBOHF regulation involves direct binding of  $Ca^{2+}$  to its EF-hands and  $Ca^{2+}$ -dependent phosphorylation by CBL1/9–CIPK26 complexes [95]. Direct binding of  $Ca^{2+}$  to EF-hand motifs on MAMP-induced elevation of  $[Ca^{2+}]_i$  causes conformational changes and activation of RBOHD [96]. Additionally, RBOHD is activated by protein phosphorylation at multiple sites via calcium-dependent protein kinase 5 (CPK5) [97,98] and MAMP-receptor-associated Botrytis-induced kinase 1 (BIK1) [53]. In addition to local defences,  $Ca^{2+}$  and

RBOHD-dependent ROS production is implicated in the systemic signal propagation required for long-distance signalling [98–100]. In accordance with the current model [98], the perception of MAMPs triggers a rapid rise of  $[Ca^{2+}]_i$ , causing the activation of CPK5 and subsequent phosphorylation of RBOHD and other CPK5 substrates. Apoplastic  $H_2O_2$ , generated after dismutation of the  $O_2^-$  produced by the RBOHD, probably represents the cell-permeable signal, which serves as the stimulus for further reiterations of calcium-dependent CPK5 activation and RBOHD phosphorylation, resulting in rapid propagation of the MAMP signal throughout the plant.

### 2.3.2. MAPK signalling

Plant mitogen-activated protein kinase (MAPK) cascades generally comprise MAPKK kinases (or MEKKs), which receive signals from receptors/sensors and phosphorylate downstream MAPK kinases (or MKKs) and which subsequently activate MAPKs (or MPKs) that control the activities and synthesis of a plethora of transcription factors (TFs), enzymes, hormones, peptides and antimicrobial chemicals [101,102]. In *Arabidopsis*, two kinase cascades, MKK4/MKK5–MPK3/MPK6 [103] and MEKK1–MKK1/MKK2–MPK4, [104] are activated after perception of MAMPs/DAMPs. The activation of MEKK1–MKK1/MKK2–MPK4 negatively regulates ROS and salicylic acid (SA) production [105,106], as well as repressing cell death and immune responses [107]. MKK4/MKK5–MPK3/MPK6 cascade positively regulates the expression of several defence-related genes [60,103] and promotes accumulation of camalexin via transcription factor WRKY33 [108,109]. Moreover, activation of MPK3/MPK6 is required for full priming of stress responses [110] and increases ethylene production via ACC synthases ACS2/ACS6 [111]. Given the essential nature of the MKK4/MKK5–MPK3/MPK6 cascade, its activation has to be precisely controlled, since inappropriate activation (e.g. constitutively activated MKK4/MKK5 [112] or over-expression of MPK3 [113]) may promote hypersensitive response (HR)-like cell death or be lethal to plants. MPK3 has also recently been indicated to be a negative regulator of defence gene expression, flg22-triggered SA accumulation and disease resistance to *Pseudomonas syringae* [114]. Another negative regulator of MAPK activities is MAPK phosphatase 2 (MKP2), which interacts with and dephosphorylates MPK3 and MPK6 [115]. Additionally, a Raf-like MAPKK kinase (EDR1) has been proposed to negatively regulate the MKK4/MKK5–MPK3/MPK6 cascade by physically interacting with MKK4 and MKK5 via its N-terminal domain [113].

## 2.4. Transcriptional reprogramming converges with complex phytohormone signalling networks

Transcription factors (TFs) involved in plant immunity reside in transcriptional complexes and, together with co-regulatory proteins, directly or indirectly recruit RNA polymerase II to the target promoters or release it from them [116]. TFs vital for plant immunity comprise members of the AP2/ERF, bHLH, bZIP, MYB, NAC and WRKY TF families and perform diverse roles [9]. For instance, certain members of apetala2/ethylene-response element binding factor (AP2/ERF) participate in the regulation of genes related to the jasmonic acid (JA) and

ethylene hormone signalling pathways [117]. *AtMYC2/JAI1/JIN1* and closely related proteins *AtMYC3* and *AtMYC4* belong to basic-helix-loop-helix (bHLH) TFs and coordinate JA-mediated defence responses with other phytohormones (salicylic acid (SA), abscisic acid (ABA), gibberellins (GA) and auxin) [118]. TGA/basic domain leucine zipper (bZIP) family members are central players in SA-mediated resistance to biotrophic pathogens. Moreover, *AtTGA2*, 5 and 6 TF have central roles in establishing systemic acquired resistance (SAR), regulate host detoxification pathways and are essential activators of certain ethylene-induced defence responses [119].

TF expression and activities are regulated in multiple ways. Certain nucleotide-binding site/leucine-rich repeat (NLR) proteins directly regulate transcription by physically interacting with TFs [120–123]. Several TFs are controlled by phosphorylation as downstream targets of activated MAPK cascades [109,124–127]. Another mechanism of TFs activation is carried out by  $\text{Ca}^{2+}$  sensors such as CaMs and CDPKs [128–133]. Additional factors (e.g. components of mediator complex [134,135], chromatin modifications [136–138]) and levels of regulation (e.g. ubiquitination [139], sumoylation [140], alternative mRNA splicing [141]) also contribute to the complexity of transcriptional networks and fine-tuning of immune responses.

Coordination of diverse stress responses and growth is resolved within complex phytohormone signalling networks, in which salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) perform central roles, while other hormones merely modulate their responses [142]. SA is synthesized in chloroplasts from chorismate by isochorismate synthase [143] and exported to the cytosol [144]. In response to pathogens and various abiotic stresses, SA mediates expression of pathogenesis-related (PR) genes and the synthesis of antimicrobial compounds to provide basal defence and systemic acquired resistance (SAR) [145]. SA activates these defence responses through transcription cofactor nonexpresser of PR genes 1 (NPR1) [146] and transcription factors TGA2, TGA5 and TGA6 [119]. In the absence of SA, NPR1 is sequestered in the cytosol and forms oligomeric complexes stabilized by intermolecular disulphide bonds [147]. In response to activation of SA pathway, thioredoxins reduce these disulphide bonds, causing the release of NPR1 in monomeric form, which can translocate to the nucleus via a nuclear translocation signal (NLS) [148]. In addition, NPR1 protein levels oscillate through CUL3<sup>NPR3</sup>- and CUL3<sup>NPR4</sup>-mediated degradation in the nucleus, which is required for fine-tuning of immune responses [147,149,150].

Jasmonates (JAs) are plant hormones with essential roles in plant defence and development [118]. JAs are derived from  $\alpha$ -linolenic acid liberated from membrane phospholipids by the action of phospholipase A and enzymatically converted in a series of steps in chloroplasts and peroxisomes, to be finally transformed into bioactive molecule JA-isoleucine (JA-Ile) in the cytosol [151]. JA-signalling is activated after repressor removal [152,153]. In unstimulated cells, jasmonate ZIM domain (JAZ) proteins repress transcription of JA signalling components, such as the basic-helix-loop-helix (bHLH) master transcription factor MYC2 and its close homologues MYC3 and MYC4 [154]. On JA signal perception by coronatine insensitive 1 (COI1), a component of the Skp1-Cul-F-box protein (SCF) E3 ligase complex, JAZ repressor proteins are targeted for proteasome-mediated degradation and MYC2 activates the transcription of several JA-responsive genes [154,155].

Ethylene (ET) is a gaseous hormone that often works synergistically with JA [156]. Important steps in ET biosynthesis are the conversion of S-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and oxidation of ACC by ACC oxidase to form ET [157]. In *Arabidopsis*, MPK3 and MPK6 phosphorylate ACS2 and ACS6 proteins to prevent rapid degradation of ACS2/ACS6 by the 26S proteasome pathway and enhance ET production in response to MAMP perception [111]. MPK3 and MPK6 also phosphorylate TF WRKY33, which subsequently binds to ACS promoters and regulates ET production [158]. Generated ET binds to its receptors, such as ethylene response 1 (ETR1) in the ER membrane. When ET is absent, active receptors ETR1 negatively regulate ethylene insensitive2 (EIN2) through phosphorylation via Raf-like protein kinase constitutive triple response1 (CTR1) [159]. At the same time, TFs ethylene insensitive3 (EIN3) and EIN3-like1 (EIL1) are recruited by two F-box proteins, EBF1 and EBF2, to 26S proteasomal degradation. On ET signal perception, the ETR1 receptors are inactivated and CTR1 repressed. Subsequently, the C-terminal part of EIN2 is cleaved and translocated to the nucleus [160]. This induces degradation of EBF1 and EBF2 and stabilizes EIN3 and EIL1, which regulate expression of ET-responsive genes (e.g. TF ERF1 and ORA59) [161,162].

Plant hormonal crosstalk is extensive and occurs in several combinations [163]. The molecular mechanism underlying SA-mediated reprogramming of the JA transcriptional network points to immune signalling antagonism and the involvement of transcriptional regulators NPR1, TGA, WRKY and ORA59 as signal integrators [164]. Phytohormones JA and ET synergistically regulate plant defence responses to necrotrophic fungi via JA-induced EIN3 and EIL1 activation and ET-induced EIN3 and EIL1 stabilization. In addition, antagonistic effects observed in JA and ET signalling are mediated by the interaction of JA-activated MYC2 TF and ET-stabilized TF EIN3 [165].

## 2.5. Inactivation of immune signalling pathways

Various negative regulation mechanisms ensure immune signalling activation is switched off when there is no danger. In the absence of ligand, several phosphatases interact with PRRs and their associated kinases to keep immune complexes inactive through dephosphorylation. For instance, Ser/Thr phosphatase type 2A (PP2A) constitutively associates with BRI1-associated kinase1 (BAK1) and negatively controls BAK1 activation in PRR immune receptor complexes [166]. Negative regulation exerted by downstream phosphatases is illustrated by *Arabidopsis* MAPK phosphatase 1 (MKP1) operating as a negative regulator of MPK6-mediated MAMP responses [167] and also observed with MAPK phosphatase 2 (MKP2), which acts as the key regulator of MPK3 and MPK6 networks controlling both abiotic and specific pathogen responses in plants [115].

Ubiquitination and proteasomal degradation are other mechanisms by which plant immune responses are attenuated. For example, two U-box E3 ubiquitin ligases, PUB12 and PUB13, are recruited to flagellin-induced FLS2/BAK1 receptor complex and phosphorylated by BAK1 to polyubiquitinate FLS2 and promote its degradation [168]. Downregulation of immune signalling can also be achieved by ligand-induced endocytosis and degradation [169]. This has

been reported in localization studies of flg22-induced FLS2 receptors in *Arabidopsis* [170] and was recently proposed as a mechanism for desensitizing host cells to flg22 stimulus and in turning over ligand-bound FLS2 [171].

### 3. Plant defence responses to xylem-invading pathogens

#### 3.1. General perception of MAMPs in roots

Despite the fact that roots are subjected to a rich microbial community, the perception of MAMPs and immune responses in roots are poorly understood. Millet et al. [172] studied immune responses in *Arabidopsis* roots after exposure to flg22, PGN and chitin. Flg22 and PGN initiated signalling only in association with LRR RLK BAK1. Furthermore, bacterial flg22 and PGN triggered a localized response in the elongation zone of the root tip, while chitin induced a response only in the mature zones of roots. It is thus likely plants have evolved tissue-specific MAMP-triggered immune responses, depending on the nature of the attacker [172,173]. While fungi and nematodes can directly penetrate the epidermal layer of roots, bacteria cannot and therefore exploit the weakest part of the roots as infection site. This hypothesis has been confirmed by recent FLS2 expression studies [174], which indicated that basal FLS2 promoter activity is restricted to the vascular cylinder and outgrowing lateral roots. Moreover, the FLS2 receptor system in roots is functional, since flg22 treatment induced rapid calcium influx and caused phosphorylation of MAPK [174]. Whole transcriptome expression analysis of flg22-elicited roots also revealed a set of genes specifically upregulated in roots, with functions in hormone and stress signalling, root and lateral root development, signalling and defence [174].

#### 3.2. Perception of vascular wilt pathogens

At early stages of infection, vascular wilt pathogens are faced with preformed physical and chemical root defences and MAMP-induced immune responses that hinder their invasion [20]. Once they breach the rigid secondary xylem walls and enter the xylem vessels, vascular wilt pathogens are presumably recognized by specific extracellular receptors in the parenchyma cells surrounding the xylem vessels [21].

##### 3.2.1. Perception of *Verticillium* spp.

In tomato, extracellular LRR RLP Ve1 [24,175,176] plays a role in xylem defence and provides resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* [177,178]. In recent years, several other homologue genes have been reported in *Gossypium*, *Solanum* and *Mentha*. A functional *Ve1* orthologue has also been discovered in *Nicotiana glutinosa* [179]. Ve1 recognizes a small effector protein, Ave1, with a similarity to plant natriuretic peptides involved in regulation of water and ion homeostasis [180]. Phylogenetic analysis has indicated hundreds of Ave1 homologues in plants but only a few in fungi, suggesting *Verticillium* spp. acquired Ave1 through horizontal gene transfer [180].

Ve1 forms heterodimers with a tomato orthologue of the *Arabidopsis* RLK suppressor of BIR1-1/evershed (SOBIR1/EVR) in the absence of Ave1 [181]. However, Ve1-mediated signalling also requires other critical signalling components, such as SERK1 and SERK3/BAK1, to establish *Verticillium* resistance in tomato and *Arabidopsis* [177,178,182]. Additionally, Ve1-mediated signalling depends on ER-QC-assisted folding mediated by ER-resident chaperones HSP70 binding proteins (BiPs) and lectin-type calreticulins (CRTs) [183].

### 3.2.2. Perception of *Fusarium oxysporum* f. sp. *lycopersici*

Three I (immunity) genes have been identified in tomato [184] in a resistance response to *Fusarium oxysporum* f. sp. *lycopersici* (Fol) that involves callose deposition, accumulation of phenolics and formation of tyloses and gels [185]. The I-2 gene encodes an intracellular CC-NB-LRR receptor protein that perceives Fol effector protein Avr2 (secreted in xylem 3; Six3) [186]. Avr2 is under the control of transcription factor Sge1 and is highly expressed in roots and xylem vessels [187]. Avr2 forms homodimers and requires nuclear localization to trigger I-2-mediated cell death [188], which can be strongly suppressed by Fol effector Six6 [189]. It has recently been shown that Six5 also contributes to the virulence of Fol in tomato plants that Six5 and Avr2 can interact and are together required for I-2-mediated resistance [190].

### 3.2.3. Perception of *Xanthomonas oryzae* pv. *oryzae*

The rice LRR RLK Xa21 that provides resistance to *Xanthomonas oryzae* pv. *oryzae* (Xoo) [191] recognizes sulphated peptide from the N-terminal part of the secreted quorum-sensing (QS) signal molecule activator of Xa21 (Ax21) [192,193]. In the absence of a signal, Xa21 associates with XB24, a protein with ATPase activity that enhances Xa21 autophosphorylation of Ser/Thr residues [194]. On Ax21 recognition, XB24 is released and Xa21 becomes activated to induce rice defence responses [193]. Subsequently, a protein phosphatase 2C (XB15) specifically interacts with activated Xa21, leading to dephosphorylation and inactivation of Xa21 [195]. In addition, several other proteins (e.g. RING finger ubiquitin ligase XB3 [196] and plant-specific ankyrin-repeat protein XB25 [197]) are associated with Xa21 and required for Xa21 accumulation and resistance to Xoo [193]. Moreover, Xa21 binds XB10, a WRKY62 transcription factor. When overexpressed, XB10 suppresses the activation of defence-related genes *OsPR1* and *OsPR10* and acts as a negative regulator of basal and Xa21-mediated immunity [198]. Xa21 also interacts with the endoplasmic reticulum (ER) chaperone BiP3, which regulates its stability and processing [199].

### 3.2.4. Perception of *Ralstonia solanacearum*

A pair of *Arabidopsis thaliana* TIR-NB-LRR proteins, RRS1 and RPS4, function together in disease resistance against *Colletotrichum higginsianum*, *Pseudomonas syringae* pv. *tomato* and *Ralstonia solanacearum* [200–202]. RRS1 and RPS4 proteins form an inactive heterodimer complex through the SH motif in their TIR domains [5,121]. RRS1 protein recognizes and, through its C-terminal WRKY domain, directly binds *R. solanacearum* effector PopP2 [203–205]. This leads to disruption of RRS1/RPS4 TIR heterodimer (but not full-length hetero-complex), allowing the formation of signalling active RPS4 TIR homodimer. PopP2 interacts

with other WRKY domain-containing proteins and acetylates lysines to block DNA binding, suggesting that PopP2 interferes with WRKY TF-dependent defence [205,206].

### 3.3. Induced defence responses to vascular wilt pathogens

Recognition of vascular wilt pathogens by plant immune receptor complexes activates defence responses in the xylem vessels. Physical defence responses that confine pathogens from further spread comprise the formation of tyloses, accumulation of pectin-rich gels and gums, vascular coating and callose and secondary cell wall deposition [20,21]. An interesting adaptation to vascular wilt infection is vein clearing, a tissue-specific developmental programme leading to the formation of new xylem elements [207]. Furthermore, significant metabolic changes have been reported in response to xylem infection and involve the induction of pathogenesis-related (PR) proteins, peroxidases, proteases as well as the production of antimicrobial secondary metabolites such as phytoalexins, sulphur-containing compounds and phenolic compounds [20,21].

Studies of defence signalling in response to root pathogens have so far mainly focused on the leaves and have provided evidence that defence mechanisms involve similar signalling pathways (Ca<sup>2+</sup>-signalling, induction of ROS and MAPK cascades, modulation of phytohormone signalling) [20]. Moreover, plant microarray and RNASeq studies have revealed that the interaction between vascular wilt pathogens and host plants involves transcriptional reprogramming of hundreds of genes [208–211]. Interestingly, in an incompatible interaction, only modest changes in gene and protein expression have been reported [210,212–214] and most of the differentially expressed genes have been repressed in roots rather than in leaves [211]. Moreover, genes implicated in photorespiration, hypoxia, glyoxylate metabolism and auxin signalling show inverse regulation on infection with the foliar pathogen *Cladosporium fulvum* or root pathogen *Verticillium dahliae* [210].

Genome-wide analyses on transcriptional and proteomic levels, together with functional characterization of individual genes, have revealed a convergence of signalling pathways in response to individual pathogens, in mostly controlled conditions. In the field, plants are simultaneously challenged by multiple stress factors, both biotic and abiotic. Even though signalling components of plant regulatory networks are partly shared in both and point to general stress response mechanisms, there is evidence of specific responses to combined stresses that are controlled by different signalling pathways and such studies may provide additional candidates for crop protection breeding [14–16,18].

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# **Absciscic Acid Signalling as a Target for Enhancing Drought Tolerance**

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Additional information is available at the end of the chapter

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## **Abstract**

Absciscic acid (ABA) is a vital hormone that confers abiotic stress tolerance in plants. The identification of PYR/PYL/RCAR proteins as bona fide ABA receptors and the subsequent elucidation of the structural mechanisms of the core ABA signalling pathway in recent years has provided new and powerful insights in targeting ABA signalling to enhance abiotic stress tolerance in agriculture. This chapter reviews the components and molecular mechanisms of the core ABA signalling pathway, as revealed by X-ray crystallography studies, and how these knowledge led to preliminary efforts in novel biotechnological developments to improve stress tolerance in plants.

**Keywords:** Absciscic acid signalling, ABA receptors, PYL/PYR/RCAR, abiotic stress tolerance

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## **1. Introduction**

The persistence of drought and climate change continues to cause heavy crop losses worldwide and pose a threat to the global food security. To meet the demands of a booming global population, the World Bank estimates that food production must increase by at least 50% in the year 2050 [1]. Increasing agricultural productivity is one practical solution to the food crisis, as has been demonstrated by the Green Revolution, which was estimated to have saved more than one billion people from famine [2]. However, such intensive farming can leave harmful impacts on the environment, such as land degradation and freshwater depletion. Agriculture

is the largest consumer of global freshwater resources [3], and it is predicted that in the year 2030, the world will be in a 40% water shortage [4]. Therefore, in view of the water and food crises, solutions that aim to increase crop productivity while limiting agricultural water consumption are the most valuable. Such solutions may potentially arise from the fine understanding of how plants perceive and respond to abiotic stress signals.

The plant hormone abscisic acid (ABA) was first discovered in the 1960s and shortly after, its role as the central and critical regulator of abiotic stress response has become clear. Under environmental stress such as drought, ABA levels rise strongly and rapidly, triggering stress tolerant effects, such as stomatal closure, to enable the plants to conserve water and survive through the harsh condition [5]. Exogenous ABA treatment has been shown to maintain the survival and quality of plants subjected to drought stress [6]. However, the agricultural use of ABA has been limited by its short-lived bioactivity, which is due to its chemical instability and rapid catabolism in plants [7]. Therefore, there has been much interests in the development of synthetic compounds that functionally mimic ABA but exhibit longer periods of bioactivity. While earlier attempts of designing ABA analogues guided by the hormone's chemical structure and catabolic pathway had not led to any desirable candidates [8], recent approaches based on the molecular mechanisms of ABA receptor signalling has shown more promising results. Such developments have made been possible by advances in the structural mechanisms of ABA signalling components that have emerged in the past few years, as reviewed in the following sections.

## **2. ABA is a regulator of abiotic stress tolerance**

Stress signals, such as drought, salinity, and temperature extremes, trigger the biosynthesis of ABA from carotenoid precursors [9]. ABA is synthesised in vascular tissues and transported to the roots and leaves where the actions occur [10]. In leaves, ABA induces stomata closure to prevent transpirational water loss and promotes the accumulation of osmocompatible solutes to retain water [11]. ABA inhibits root and shoot growth and promotes seed dormancy. Such inhibitory effects help plants to pull through adverse conditions and germinate only when the conditions are favourable for growth. ABA also confers tolerance to freezing through the induction of dehydration-tolerance genes [12].

The effects of ABA has immense agricultural and economic value. Massive amounts of crops are lost to drought every year and the situation may worsen as climate change persists. The ability to manipulate ABA responses offers an innovative solution to alleviate crop loss and sustain agricultural yield in the face of the inevitable climatic change. Moreover, the activation of ABA responses may enhance agricultural water use efficiency, addressing concerns of the global water scarcity issue. A sound understanding of ABA signalling is critical to the successful development of approaches to manipulate ABA responses.

### 3. Components of the core ABA signalling pathway

#### 3.1. ABA receptors

“There are things known and there are things unknown, and in between are the doors of perception.” — Aldous Huxley

ABA is a messenger that carries the abiotic stress signal. The first step for the plant cells to be aware of the stress is to perceive the stress signal. The perception and transmission of the signal is carried out by ABA receptors, which recognise the ABA molecule and convey the message to downstream effectors.

Although the importance of ABA and many aspects of its signalling has been established following the discovery of the hormone in the 1960s, the identity of ABA receptors has remained elusive for almost half a century. Early efforts to identify ABA receptors employ forward genetics screens of ABA-insensitive mutants, which have instead identified several mediators of ABA signalling downstream of the receptors, such as PP2Cs and transcription factors [13, 14]. The use of alternative approaches by virtue of ABA binding has led to a number of putative candidates (FCA, CHLH, GCR2, GTG1, and GTG2) but none has been further substantiated [15]. In retrospect, these approaches had been futile for two reasons. First, the true identity of ABA receptors had been masked by genetic redundancy, and had thus eluded identification by classical forward genetic screens. Second, the identification of ABA-binding proteins had not addressed the links of the putative candidates to well-established components of ABA signalling.

The discovery of the PYR/PYL/RCAR family of proteins as bona fide ABA receptors had been different from the earlier attempts. Several groups had independently discovered this family of novel candidates, which fit elegantly into a model that connected the core components of the ABA signal transduction pathway. The first landmark studies were two reports published in *Science* in May 2009 [16, 17]. To overcome genetic redundancy issues, Park et al. [17] used a selective ABA agonist, pyrabactin, in a chemical genetic screen of mutagenised *Arabidopsis* seeds and identified *PYR1* (for pyrabactin resistance 1). *PYR1* belongs to the START domain superfamily of proteins that includes the mammalian STARD proteins. By homology, 13 other *Arabidopsis* *PYR1*-like (PYL) members were identified and named PYL1–13 (Table 1). In a different approach, Ma et al. [16] screened for interactors with the type 2C protein phosphatase (PP2C) ABI2, which is a known component of ABA signalling, and identified the same 14 START domain family members, naming them as RCAR1–14 (Regulatory Component of ABA Response) (Table 1). For simplicity, the PYR/PYL/RCAR members are referred to in this chapter as PYL proteins. Other similar studies have also identified PYL members by their interactions with PP2Cs [18, 19] and collectively, these studies showed that PYL proteins are able to bind to ABA and in the presence of ABA, PYL proteins interact with and inhibit the ABA-signalling PP2Cs.

Protein Name			Solved Structures		
PYR/PYL	RCAR	Mutation(s)	Complex with		PDB ID
			Ligand	PP2C	
PYR1	RCAR11				3K3K
			ABA		3K3K, 3K90
			Pyrabactin		3NJO
			AS6		3WG8
			ABA	HAB1	3QN1
		H60P	ABA	HAB1	3ZVU
		K59R, V81I, F108A, F159L	Mandipropamid	HAB1	4WVO
PYL1	RCAR12				3KAY
			ABA		3JRS
			Pyrabactin		3NEF, 3NEG
			ABA	ABI1	3KDJ, 3JRQ
			Pyrabactin	ABI1	3NMN
PYL2	RCAR14				3KDH, 3KL1, 3KAZ
			ABA		3KB0, 3KDI
			Pyrabactin		3NJ0, 3NMH, 3NR4, 3NS2
		V114I	Pyrabactin		3NJ1
		A93F	Pyrabactin		3NMP
		A93F	Pyrabactin	HAB1	3NMT
		A93F	Pyrabactin	ABI2	3NMV
			ABA	HAB1	3KB3
			ABA	ABI2	3UJL
			Quinabactin (AM1)	HAB1	4LG5, 4LA7
			AM2	HAB1	4LGA
			AM3	HAB1	4LGB
PYL3	RCAR13				3KLX
			ABA		4DSB, 4DSC
			Pyrabactin		3OJI
			(-)-ABA		4JDA

Members of the PYR/PYL/RCAR family

Subset of Group A PP2CS		ABA		HAB1	4DS8
	PYL4	RCAR10			
	PYL5	RCAR8	4JDL		
	PYL6	RCAR9			
	PYL7	RCAR2			
	PYL8	RCAR3			
	PYL9	RCAR1	ABA	3OQU, 3W9R	
			3UQH, 3RT2		
	PYL10	RCAR4	ABA	3R6P	
			HAB1		3RT0
	PYL11	RCAR5			
	PYL12	RCAR6			
	PYL13	RCAR7	PP2CA		4N0G
	Solved Structures				
	Protein Name		Complex with		PDB ID
			PYR/PYL/RCAR	SnRK2	
	ABI1	PYL1-ABA		3KDJ, 3JRQ	
		PYL1-Pyrabactin		3NMN	
	ABI2			3UJK	
PYL2-ABA		3UJL			
PYL2(A93F)-Pyrabactin		3NMV			
HAB1	PYR1-ABA		3QN1		
	PYR1(H60P)-ABA		3ZVU		
	PYR1(K59R, V81I, F108A, F159L)-Mandipropamid		4WVO		
	PYL2-ABA		3KB3		
	PYL2(A93F)-Pyrabactin		3NMT		
	PYL2-Quinabactin (AM1)		4LG5, 4LA7		
	PYL2-AM2		4LGA		
	PYL2-AM3		4LGB		
	PYL3-ABA		4DS8		
	PYL10		3RT0		
		SnRK2.6		3UJG	
PP2CA	PYL13		4N0G		

Subset of Group A PP2CS

	Protein Name	Solved Structures		
		Mutation(s)	Complex with	PDB ID
Subclass III SnRK2s	SnRK2.2			
	SnRK2.3	D57A, K58A		3UC3
		D59A, E60A		3UC4
		D160A		3ZUT
	SnRK2.6	D160A, S175D		3ZUU
		S7A,S29A,S43A,C131A,C137A,C159A,S166A,T176A		3UDB
			HAB1	3UJG

**Table 1.** List of members belonging to the components of the core ABA signalling pathway and their solved structures.

3.2. Group 2C Protein Phosphatases (PP2Cs)

PP2Cs are a group of monomeric Mg<sup>2+</sup>/Mn<sup>2+</sup>-dependent serine/threonine phosphatases found in virtually all organisms and are known for their roles in the regulation of cell growth and cellular stress signalling. In Arabidopsis, there are 76 known PP2Cs that are genetically clustered into 10 groups (A-J), with the exception of 6 genes that could not be clustered [20]. At least 6 of the 9 members of group A PP2Cs have been shown to be involved in ABA signalling. Among these, ABI1, ABI2, and HAB1 are the most well-studied members and are known to be the negative regulators of ABA signalling.

The *abi1* and *abi2* (ABA insensitive) mutants were isolated from genetic screens of ABA insensitive mutants [21-24]. HAB1 was subsequently identified by homology to ABI1 and ABI2 [25]. The *abi1-1* (ABI1 G180D), *abi2-1* (ABI2 G168D), and *hab1* (HAB1 G246D) mutants displayed dominant ABA insensitive phenotypes, which are later known to be attributed to the loss of regulation by PYL proteins. The isolation of additional recessive loss-of-function mutations that resulted in ABA hypersensitive phenotype provided the early evidence that PP2Cs are negative regulators of ABA signalling [26-28]. This was further supported by the observations that double- or triple-PP2C-knockout mutants displayed enhanced ABA responses while constitutive expression of HAB1 led to reduced ABA sensitivity [28-30].

3.3. Snf1-related protein kinases 2 (SnRK2s)

While members of PP2Cs are known to play a negative regulatory role in ABA signalling, it is not surprising that a group of protein kinases are conversely the positive effectors. The Arabidopsis Snf1-related protein kinase (SnRK) group of kinases share a high degree of homology with the yeast Snf1 and catalytic subunits of mammalian AMPK. There are three classes of SnRKs in Arabidopsis, namely SnRK1, SnRK2, and SnRK3, which comprises of 3, 10, and 25 members, respectively [31]. SnRK2s are further divided into subclasses I, II, and III. While members of subclass I are not responsive to ABA, subclass II members are weakly

activated by ABA. All three members of subclass III, namely SnRK2.2/SRK2D, SnRK2.3/SRK2I, and SnRK2.6/SRK2E/OST1, are strongly activated by ABA and are known to be the main positive regulators of ABA signalling. SnRK2.6 is known for its role in stomata regulation in guard cells [32, 33], while SnRK2.2 and SnRK2.3 are important in the inhibition of seed germination and root growth [34]. Consequently, triple mutants lacking SnRK2.2, SnRK2.3, and SnRK2.6 are deficient in almost all ABA responses, indicating that class III SnRK2s play a central role in the core ABA signalling pathway [35-37].

Active SnRK2s are autophosphorylated and are able to directly phosphorylate target proteins such as ion channels and transcription factors to elicit the ABA response. ABA-induced stomata closure is mediated by the effects of SnRK2.6 on ion channels. Under ABA stimulation, SnRK2.6 activates the slow-type anion channel SLAC1 and inhibits the inward-rectifying potassium channel, KAT1, by the phosphorylation of these ion channels, resulting in stomata closure [38-40].

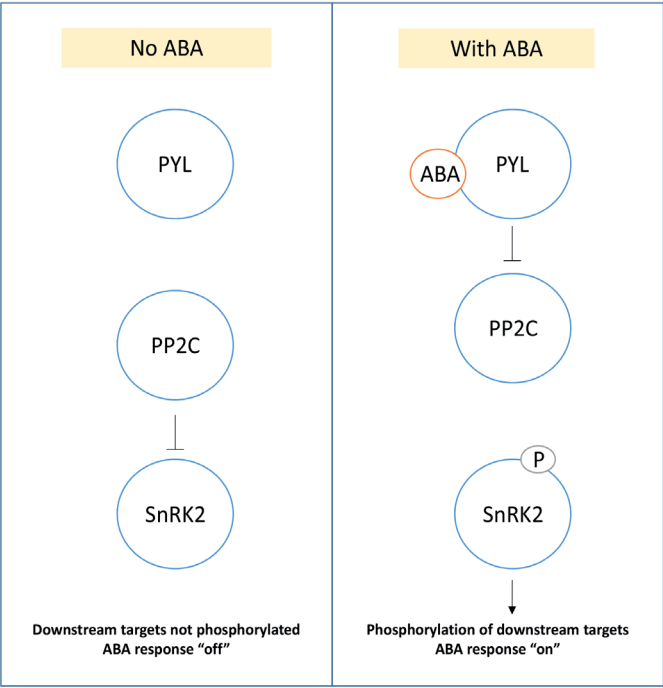
ABA induction of target gene expression is mediated by the SnRK2 phosphorylation of transcription factors known as ABRE-binding (AREB) proteins or ABRE-binding factors (ABFs). The AREB/ABFs are basic domain leucine zipper (bZIP) transcription factors that recognises the ABA-responsive elements (ABREs) located in the promoters of ABA-responsive genes. Of the nine AREB/ABF homologs found in *Arabidopsis*, the AREB1/ABF2, AREB2/ABF4, and ABF3 were found to be master transcription factors responsible for regulating the ABRE-dependent expression of stress-responsive genes [41-44]. Such genes include Late Embryogenesis Abundant (LEA)-class genes, transcription factors, and mediators of ABA signalling.

## 4. Structural mechanisms of ABA receptor signalling

The core ABA receptor signalling cascade is comprised of the PYL ABA receptors, PP2Cs and SnRK2s (Figure 1). In the absence of ABA, PP2Cs such as ABI1, ABI2, and HAB1 inhibit the activities of SnRK2s, thus silencing the ABA response. During abiotic stress, ABA is generated rapidly and binds to the PYL proteins. ABA binding induces conformational changes to the PYL proteins, allowing the activated receptors to interact with and inhibit the PP2Cs. Consequently, the SnRK2s are relieved of inhibition by PP2Cs and are autoactivated by autophosphorylation. Active SnRK2s are able to phosphorylate their targets such as ion channels and AREB/ABF transcription factors to activate the ABA responses. Reconstitution of the core ABA signalling pathway has been demonstrated by co-expression of the core components (PYL, PP2C, SnRK2, and ABF) in plant protoplasts [45]. The following subsections will explain in detail the molecular mechanisms of each step of the core ABA signalling pathway.

### 4.1. Structure and properties of ABA receptors

To date, the structures of eight out of 14 PYL members have been solved either in their apo- or ligand-bound forms or in complexes with PP2C (Table 1). The overall PYL structures exhibit the helix-grip fold, a hallmark of START domain/Bet v 1-fold proteins, which is characterised



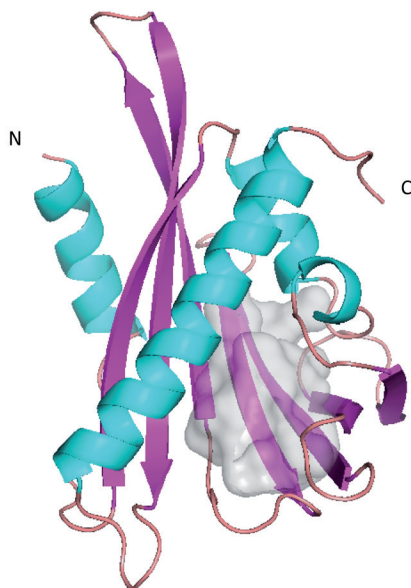
**Figure 1.** Summary model of the core ABA signalling pathway.

by the presence of a central  $\beta$ -sheet surrounded by N- and C-termini  $\alpha$ -helices, with a long C-terminal  $\alpha$ -helix packing tightly against the  $\beta$ -sheet (Figure 2). The helix-grip fold creates a large cavity constituting the ligand binding pocket.

Static light scattering and ultracentrifugation experiments showed that the apo PYR1, PYL1, and PYL2 are dimers in the solution, while PYL4–10, except for the untested PYL7, are monomers [46]. PYL3 exist in a monomer-dimer equilibrium. Consistently, the crystal structures of apo PYR1 and PYL1–3 revealed a cis-homodimer arrangement, with the two molecules associated in parallel orientation at their pocket entrance, thus hindering ligand entry [47–52]. Homodimeric receptors dissociate into monomers upon ABA binding. PYL3 has been shown to form a trans-homodimer intermediate that is able to bind ABA and dissociate into monomers more easily, consistent with its observed mixed monomer-dimer distribution [52]. While the dimeric receptors require ABA for their activity, the monomeric PYL members are able to inhibit PP2Cs constitutively in the absence of ABA, suggesting that receptor monomerisation is crucial for PP2C inhibition [46]. Despite the ABA-independent activity, it is important to note that the interactions of monomeric PYLs with PP2Cs are greatly enhanced



in the presence of ABA [16, 19, 46]. PYL13 is a divergent member that play a unique role in ABA signalling by its ability to heterodimerise with other PYL members and antagonise their activities [53]. While it was originally thought that PYL13 selectively inhibits PP2CA in an ABA independent manner [53, 54], recent evidence showed that PYL13 inhibits ABI1, ABI2, and PP2CA with the requirement of ABA [55].



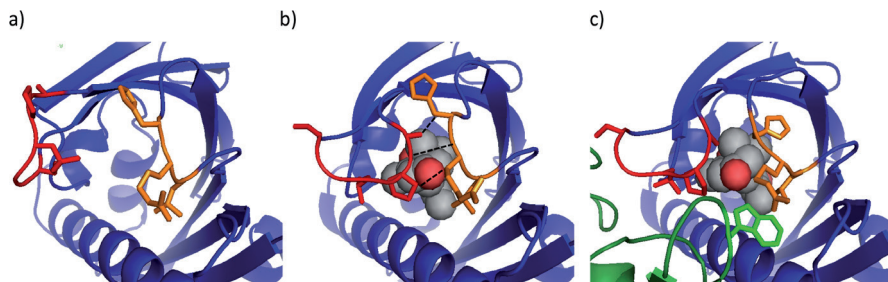
**Figure 2.** Structure of the ligand-free PYL2 ABA receptor (PDB code: 3KAZ) exhibiting the helix-grip fold. The ligand binding pocket is shown as surface presentation in grey.

#### 4.2. ABA binding

The structures of PYL bound to ABA revealed a number of features in the ABA recognition. The entrance of the ligand pocket is surrounded by two functionally important  $\beta$ -loops that are known as the "gate" and "latch" loops [47] (alternatively named Pro-Cap and Leu-Lock [49], CL2, and CL3 [51], and the  $\beta$ 3– $\beta$ 4 and  $\beta$ 5– $\beta$ 6 lid loops [48, 50]), which contain the conserved amino acid sequences SGLPA and HRL, respectively. In the structure of the apo receptor, the gate loop appears to be in an open conformation to allow ligand access (Figure 3a). In the ABA-bound structure, the gate is in a closed conformation, making contact with the latch residues (Figure 3b).

Within the ligand pocket, ABA interacts with the receptor residues through a network of charged interactions, hydrogen bonds, and hydrophobic interactions [47–51]. ABA is anchored to the inner end of the ligand pocket by a direct charged interaction between its carboxylate group

and a conserved lysine residue (K59, K86, and K64 in PYR1, PYL1, and PYL2, respectively). Nearer to the outer end of the pocket, the cyclohexene ring of ABA interacts with the receptor gate and latch residues, thus pulling the gate loop into a closed conformation (Figure 6a).



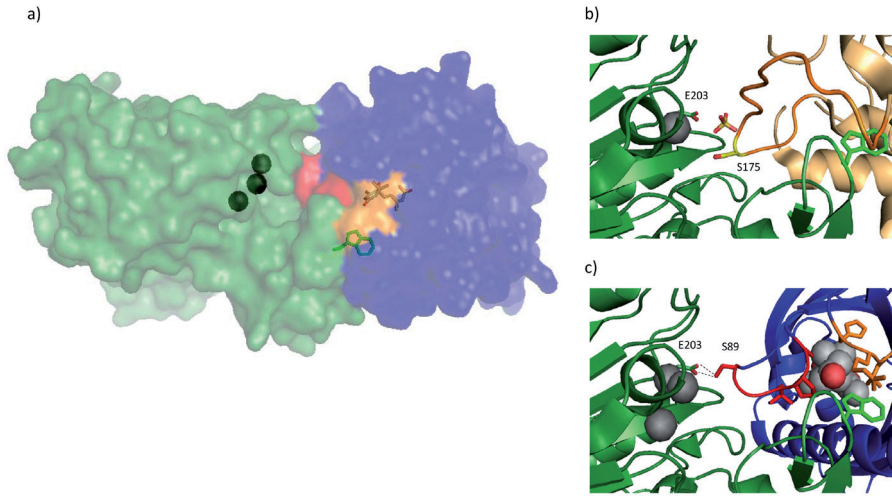
**Figure 3.** A gate-latch-lock mechanism of ABA sensing and signal relay by the PYL ABA receptor. a) The empty pocket of the ligand-free ABA receptor, represented by apo PYL2 (PDB code: 3KAZ), is guarded by a latch loop (shown in orange) and an open gate loop (shown in red). b) Structure of PYL2-ABA (PDB code: 3KB0) showing that ABA binding induces closure of the gate onto the latch loop. ABA is shown in sphere model. c) PP2C (shown in green) binding inserts a conserved tryptophan to “lock” the closed receptor gate and latch in a stable conformation, shown by the structure of the PYL2-ABA-HAB1 complex (PDB code: 3KB3). In all panels, PYL2 is shown in blue with its gate loop in red and latch loop in orange.

### 4.3. Signal relay to PP2Cs

The closure of the receptor gate upon ABA binding is functionally important for its signal transmission to the PP2Cs. While PP2Cs do not undergo obvious conformational changes in their interactions with PYLs, the ABA-induced receptor gate closure creates the necessary PYL conformation for PP2C interaction. The ABA-bound PYL protein interacts with PP2C at its closed gate and latch interface (Figure 4). In this interaction, the PP2C inserts a conserved tryptophan indole ring into the receptor pocket, forming a water-mediated network of hydrogen bonds with the receptor gate and latch residues, as well as with the ketone group of ABA (Figure 3b). The PP2C tryptophan thus acts as a molecular lock that further stabilises the receptor gate and latch interactions. This explains the observations of the marked increase in ABA binding affinities of PYLs in the presence of PP2Cs [16, 19, 48, 51]. The formation of the PYL-ABA-PP2C complex inhibits the PP2C activities in two ways. First, the PYL-ABA interacts with the PP2C at the phosphatase catalytic region (Figure 4a), thus competitively blocking substrate (SnRK2) access [56]. Second, a catalytic glutamate residue of the PP2C (E203 in HAB1) is bonded to the serine residue of the PYL gate loop (Figure 4b and 4c), thus impairing the phosphatase catalytic activity [57].

### 4.4. PP2C inhibition of SnRK2s

In the absence of ABA, PP2Cs bind to and inhibit SnRK2s. The solved structure of the SnRK2.6-HAB1 complex together with biochemical data has provided insights into the mechanisms of how PP2Cs inhibit SnRK2s [56]. In this structure, the phosphatase-kinase interaction occur



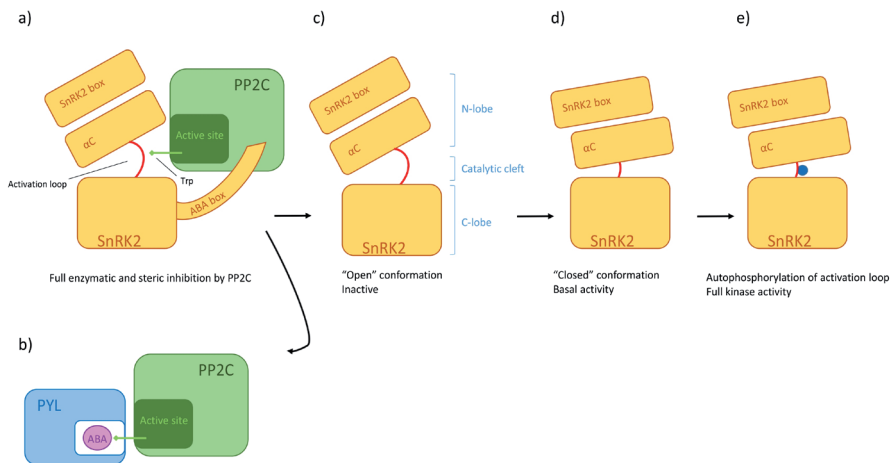
**Figure 4.** Dual mode of PP2C inhibition by the activated ABA receptor. a) Structure of PYL2-ABA-HAB1 complex (PDB code: 3KB3) in surface presentation showing the steric blocking of PP2C (shown in green, catalytic site marked by  $Mg^{2+}$  ions in grey balls) by ABA-bound PYL2 (shown in blue, with its gate and latch in red and orange respectively). ABA is shown in stick model to indicate the ligand pocket. b) Partial structure of the HAB1-SnRK2.6 complex (PDB code: 3UJG), focusing on the catalytic sites of both components. In the phosphatase reaction, a serine residue (S175 of SnRK2.6) in the activation loop of the SnRK2 (SnRK2.6 shown in light orange, with its activation loop in darker orange) is dephosphorylated. A sulphate molecule, mimicking the cleaved phosphate, is shown in stick model. This catalysis requires the PP2C catalytic glutamate (E203 of HAB1) to polarise a water molecule, enabling its nucleophilic attack on the phosphorylated S175 [57]. c) Binding of PYL2-ABA to HAB1 results in the formation of a hydrogen bond between PYL2 S89 and HAB1 E203, thus catalytically inhibiting the PP2C's phosphatase activity. In all panels, the PP2C tryptophan "lock" is shown as stick model in light green.

through mutual packing of both catalytic sites (Figure 4b and 5a). HAB1 interacts with SnRK2.6 at its PYL-interaction interface and inserts its ABA-sensing tryptophan into the catalytic cleft of SnRK2.6. Unresolved in the crystal structure, extensive evidence indicated the presence of a second interaction interface formed by the highly negatively charged C-terminal ABA box of SnRK2 and a positively charged surface region of PP2C (Figure 5a). Biochemical data has shown that at low PP2C:SnRK2 molar ratio, the kinase activity is reduced but not completely abolished [56]. This partial inhibition occurs by enzymatic dephosphorylation of a critical serine residue in the kinase activation loop. At stoichiometric levels, full inhibition is achieved through the mutual packing of catalytic sites as shown by the crystal structure.

#### 4.5. Autoactivation of SnRK2s

The understanding of how PP2Cs inhibit SnRK2s has provided partial explanations to how SnRK2s gain catalytic activity. PP2C interact with both PYL and SnRK2 at its catalytic region, suggesting that the inhibition of SnRK2 is competed away by active PYL interacting at the same PP2C interface (Figure 5a and 5b). Further biochemical and structural analyses has furnished the complete mechanisms of SnRK2 activation [58-60]. Biochemical data showed

that phosphorylation of a serine residue in the SnRK2 activation loop (S177/176/175 in SnRK2.2/2.3/2.6, respectively) is critical for full kinase activity [61]. Kinases with this serine mutated to alanine are non-phosphorylatable at this position and showed marked decrease, but not complete abolishment of kinase activity, suggesting that unphosphorylated SnRK2s have basal kinase activity [58]. Crystal structures of unphosphorylated SnRK2s have been solved in their active and inactive states, providing structural insights into the mechanisms of the basal activity [58]. The SnRK2 structures revealed a canonical bilobal kinase fold, with a well-ordered SnRK2 box packing closely in parallel to the  $\alpha$ C-helix in the N-terminal lobe (schematically illustrated in Figure 5). The structure of the active SnRK2 adopted a closed conformation, with its N-terminal lobe positioned closer to the C-terminal lobe (Figure 5d), whereas that of the inactive SnRK2 exhibited an open conformation (Figure 5c) resembling the structures of active and inactive Snf1 kinases, respectively. The basal kinase activity is attained by the closing of the catalytic cleft, which is regulated by the SnRK2 box interactions with the  $\alpha$ C-helix. The SnRK2 box mediates positioning of the  $\alpha$ C-helix to form a network of interaction that favours the binding of  $Mg^{2+}$  and ATP in the catalytic cleft. Such a positioning enables full kinase activity to be achieved upon autophosphorylation of the activation loop, which can occur intramolecularly or intermolecularly (Figure 5e).



**Figure 5.** Mechanisms of kinase activation. a) In basal state, PP2C inhibit SnRK2 by enzymatic dephosphorylation of the kinase's activation loop serine, as well as steric inhibition by physically binding to the kinase's catalytic cleft. The two PP2C-SnRK2 interaction interfaces are shown. First is through the mutual packing of active sites as indicated by the kinase's activation loop and the phosphatase's ABA-sensing tryptophan "lock". Second is through the SnRK2's C-terminal ABA box region. b) When activated by ABA, PYL compete with SnRK2 for PP2C interaction at the same PP2C catalytic region. c) and d) When relieved of PP2C inhibition, unphosphorylated SnRK2s spontaneously adopt inactive and partially active states by the opening and closing of the catalytic cleft, mediated by contacts between the SnRK2 box and  $\alpha$ C-helix. e) The closing of the catalytic cleft produces interactions that facilitates the autophosphorylation of the activation loop serine residue, which is required to attain full kinase activity.

## 5. Emerging agricultural biotechnology targeting ABA receptor signalling

The understanding of how plants perceive stress provides the opportunity to develop novel solutions to promote crop survival by boosting stress responses during adverse conditions such as water shortage. As water conservation is one of the physiological effects of ABA, the activation of ABA signalling may also promote water use efficiency in agriculture. The world is facing a crisis of freshwater shortage and the agricultural sector is the largest consumer of the global freshwater resources. Thus, the ability to manipulate ABA responses has immense value, promoting both water conservation and food productivity. ABA responses may be manipulated by transgenic approaches involving the overexpression of the effectors of ABA signalling, the structure-guided development of high efficacy agonists, or a combination of these methods.

### 5.1. Transgenic approach

Having understood the roles and mechanisms of the key players in the core ABA signalling, one approach to enhance ABA sensitivity is to generate transgenic plants overexpressing the effectors of ABA signalling. While the dimeric PYL members, PYR1, PYL1, and PYL2 require ABA for their activation, the monomeric receptors, PYL5-10, show ABA-independent constitutive activity which is greatly enhanced in the presence of ABA [46]. Thus, the constitutively active monomeric receptors are thought to be suitable candidates for transgenic overexpression to enhance ABA sensitivity. This idea is supported by the observation that transgenic *Arabidopsis* overexpressing PYL5 showed enhanced ABA sensitivity and increased drought stress tolerance [19]. Similar results have been shown in transgenic *Arabidopsis* overexpressing PYL13 [53], which has shown ABA independent inhibition of PP2CA [54]. In rice (*Oryza sativa*), the constitutive expression of OsPYL, the rice orthologue of *Arabidopsis* PYL, has also shown to improve drought and salt stress tolerance [62].

With the known structural mechanisms, genetic modifications of PYL may be incorporated to further enhance their activity. The constitutive expression of a PYL2 mutant designed to stabilise PYL-PP2C interactions has shown increased ABA signalling in transgenic *Arabidopsis* seeds [63]. However, the expression of the mutant receptor has not been detected in vegetative tissues, thus precluding further analyses in this study. In another study, overexpression of a mutant PYL4 receptor (PYL4<sup>A194T</sup>), which showed ABA-independent inhibition of PP2CA *in vitro*, resulted in enhanced drought tolerance in the transgenic plants [64]. However, as ABA is a negative growth regulator, a drawback of constitutively activating ABA responses is the impairment of growth under normal conditions, thus affecting overall yield. Approaches that allow inducible activation of ABA responses only under stressful conditions may be useful to overcome this problem.

Alternatively, the heterologous expression of SnRK2s of wheat (*Triticum aestivum* L.), TaSnRK2.4, and TaSnRK2.8 in *Arabidopsis* has been tested and the transgenic plants showed enhanced tolerance to drought, salt, and cold stresses [65, 66]. Similarly, transgenic *Arabidopsis* overexpressing the maize SnRK2 orthologue, ZmSAPK8, exhibited increased tolerance to salt stress [67]. In these studies, the heterologous expression of members of the SnRK2s did

not seem to retard the normal plant growth under unstressed conditions, thus appearing to be a potential approach for the development of stress-tolerant transgenic crops.

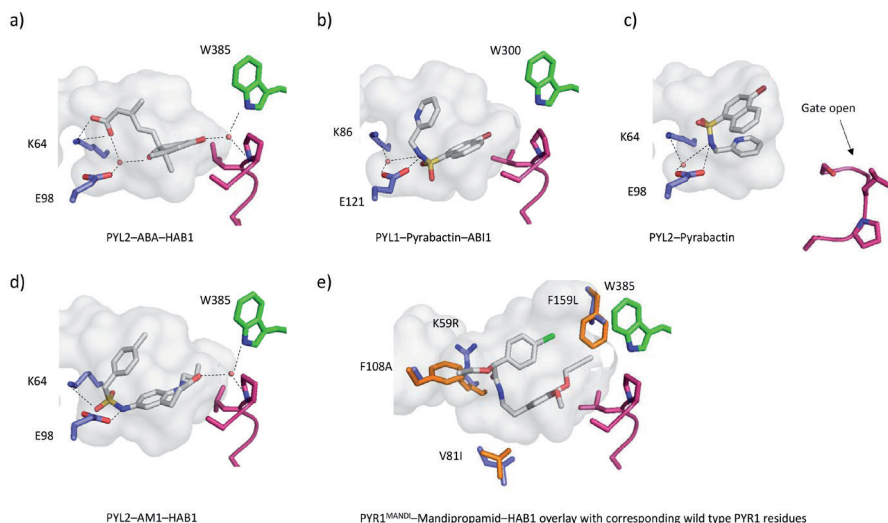
## 5.2. Agonist design

The direct application of ABA in the fields has been shown to promote drought stress tolerance. However, the widespread use of ABA in agriculture is limited due to its chemical instability and difficulty to synthesise. Therefore, there has been much research focus on the development of ABA analogues that are able to elicit the effects of ABA and exhibit better chemical stability.

Pyrabactin is a synthetic seed germination inhibitor that mediates a selective part of ABA's activities. Being a selective ABA agonist, pyrabactin has been used to overcome the problems of genetic redundancy in the identification of ABA receptors [17]. Although the direct application of pyrabactin is not practical for agriculture use, as its effects are most strongly in seeds rather than in vegetative tissues, the study of ABA receptors bound to pyrabactin has provided insights into the design of ABA receptor agonists. While ABA is a pan-agonist of all PYL members, pyrabactin selectively activates few members, including PYR1 and PYL1. Conversely, pyrabactin acts as an antagonist in PYL2. Comparisons between the structures of ABA-bound PYL, pyrabactin-activated PYL and pyrabactin-antagonised PYL complexes have revealed the ligand-receptor interactions that are important to induce the closed gate conformation necessary for receptor activation [68-71]. In the pyrabactin-activated receptor (PYR1 or PYL1) structures, pyrabactin interacts with receptor pocket residues in a similar manner as ABA, with its sulphonamide group forming a water-mediated hydrogen bond with the conserved lysine residue and its naphthalene double ring interacting with the receptor gate residues, producing the close-gate conformation (Figure 6b). These features provided a framework for the development of novel ABA agonists. On this basis, Melcher et al. screened virtual chemical libraries for compounds containing the naphthalene-1-sulphonamide group and computationally docked them into the known PYL structures [69]. In vitro screening of top candidates identified four compounds with efficacies comparable to that of pyrabactin.

Examination of the structure of the pyrabactin-antagonised PYL2 receptor revealed a flip in the orientation of pyrabactin relative to that in the pyrabactin-activated receptor structures (Figure 6c). In the antagonist position, the naphthalene double ring and pyridine ring are in reversed order, placing the smaller pyridine ring towards the pocket entrance in a distance too far to interact with the receptor gate. Therefore, pyrabactin antagonises PYL2 by occupying the receptor pocket while being unable to produce the closed gate conformation. With this knowledge, an agonist termed AM1/quinabactin was designed with a similar scaffold as pyrabactin but with the sulphonamide group in reversed orientation [72, 73]. The structures of PYL2 bound to AM1/quinabactin showed that the AM1/quinabactin is oriented with its double ring facing the receptor gate, producing a closed gate conformation (Figure 6d). Furthermore, AM1/quinabactin showed potent in vitro efficacies in PP2C interaction, ability to promote drought tolerance when exogenously applied to plants and higher stability than ABA when exposed to mild UV [72, 73]. Therefore, AM1/quinabactin emerged as a highly promising candidate to be further evaluated for agricultural use.





**Figure 6.** Mode of receptor binding by various ligands. Ligand binding pockets of a) PYL2-ABA-HAB1 structure (PDB code: 3KB3), b) PYL1-Pyrabactin-ABI1 (PDB code: 3NMN), c) PYL2-Pyrabactin (PDB code: 3NMH), d) PYL2-AM1-HAB1 (PDB code: 4LG5), and e) PYR1<sup>MANDI</sup>-Mandipropamid-HAB1 (PDB code: 4WVO). PYL ligand pockets are shown as surface presentation in light grey. In (a) to (d), the conserved lysine and glutamate residues that anchor the ligands to the inner end of the receptor pocket are shown as stick models in blue. In all panels, receptor gate loop residues are shown in red, while the conserved PP2C tryptophan “lock” is shown as a stick model in green. In (c), pyrabactin antagonises the PYL2 receptor, leaving the receptor gate in an open conformation. In (d), the four mutant PYR1<sup>MANDI</sup> residues are shown in blue, while the corresponding wild type residues from the PYR1-ABA-HAB1 (PDB code: 3QN1) structure are shown in orange.

### 5.3. Orthogonal receptor-ligand approach

Although the discovery of AM1/quinabactin has shown promising results, pushing a new chemical into the market can be a long and costly process as the potential effects on human health and the environment need to be thoroughly assessed. To bypass such a process, an alternative solution is to make use of currently approved agrochemicals. Park et al. has demonstrated that the ABA receptor can be engineered to be activated by existing agrochemicals [74]. This effort has identified a hexuple mutant PYR1<sup>MANDI</sup> (PYR1(Y58H/K59R/V81I/F108A/S122G/F159L)) that showed strong PP2C inhibition with the agrochemical mandipropamid at nanomolar sensitivity. To understand the mode of the orthogonal receptor-ligand interactions, the crystal structure of a quadruple mutant PYR1(K59R/V81I/F108A/F159L), which contains 4 of the 6 mutations of PYR1<sup>MANDI</sup> and yielded higher quality crystals than PYR1<sup>MANDI</sup>, has been solved in complex with mandipropamid and HAB1 (Figure 6e). The crystal structure revealed that the F108A/F159L mutations created more space in the receptor pocket to fit the larger ligand. The arginine of R59 forms a hydrogen bond with the amide carbonyl of mandipropamid, mimicking the interaction between the carboxylate group of ABA with K59 of wild type PYR1. In vivo studies of transgenic Arabidopsis constitutively express-

ing PYR1<sup>MANDI</sup> showed enhanced drought survival with mandipropamid treatment, thus demonstrating the feasibility of such an approach.

## 6. Conclusions

The identification of PYL proteins as ABA receptors has placed a crucial piece of the puzzle into the previous knowledge of the core ABA signalling pathway. As such, the early efforts in the discovery of PYL proteins and the elucidation of their structural mechanisms have been recognised as one of the breakthroughs of the year 2009 by *Science* and *Science Signaling* journals [75, 76]. Subsequently, further structural studies have rapidly emerged to provide a comprehensive understanding of the molecular mechanisms of the entire core ABA signalling pathway, which has led to preliminary developments of novel approaches targeting at ABA signalling for the agricultural enhancement of abiotic stress tolerance. Using several lines of approach, these studies have demonstrated the ability to target ABA signalling for improving stress tolerance. While several studies have shown promising data at the preliminary stage, further testing in crop plants will be necessary to evaluate their agricultural feasibility. Having progressed rapidly since the initial discovery of the PYL proteins, further breakthrough advances in the agricultural improvement of abiotic stress tolerance seems highly conceivable and is much awaited for.

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# Reactive Oxygen Species and Antioxidant Enzymes Involved in Plant Tolerance to Stress

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Additional information is available at the end of the chapter

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## Abstract

Plants are continuously exposed to several stress factors in field, which affect their production. These environmental adversities generally induce the accumulation of reactive oxygen species (ROS), which can cause severe oxidative damage to plants. ROS are toxic molecules found in various subcellular compartments. The equilibrium between the production and detoxification of ROS is sustained by enzymatic and nonenzymatic antioxidants. Due to advances in molecular approaches during the last decades, nowadays it is possible to develop economically important transgenic crops that have increased tolerance to stresses. This chapter discusses the oxidative stress and damage to plants. In addition, it reports the involvement of antioxidant enzymes in the tolerance of plants to various stresses.

**Keywords:** ROS, abiotic and biotic stress, oxidative stress, antioxidative mechanisms, tolerant plants

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## 1. Introduction

Crop yield depends on the plant's ability to adapt to different types of environmental adversities, which generally induce oxidative stress. Environmental stress induces the accumulation of reactive oxygen species (ROS) in the cells of plants, which can cause severe oxidative damage to the plants, thus inhibiting growth and grain yield. ROS are involved in processes such as growth, development, response to biotic and abiotic environmental stimuli, programmed cell death, and may act as signal transducers. Stressors, hormones, development, and other several metabolic routes can stimulate ROS production that in turn may induce other routes or act directly as defense compounds [1].

Knowledge about the oxidative mechanisms in plants may contribute to the development of plants most well adapted to the environment and resistant to pathogens. Plants have defense mechanisms against oxidative damage that are activated during stress to regulate toxic levels of ROS. Antioxidant and nonantioxidant systems are involved in ROS detoxification.

During the last decades, antioxidant enzymes have been used to develop transgenic plants that have increased tolerance to several stresses. Therefore, this chapter will address the relation between abiotic and biotic stresses and ROS generation. The ROS production, major antioxidant enzymes involved in detoxification, and defense under stresses will be described. The involvement of the antioxidant enzymes in the tolerance of plants to various stresses will be also discussed.

## 2. Crop production and stress

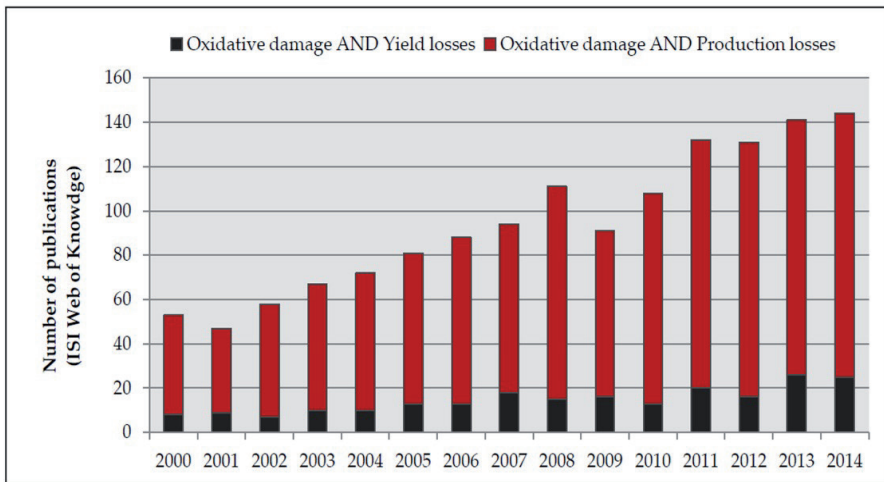
Global agricultural production has tripled in the last 50 years because of increased demand due to population growth. Genetic breeding has improved crop yields per unit area. In 1960, the food requirement per capita was approximately 2,200 kcal/day. In 2009, the global food requirement per capita increased to more than 2,800 kcal/day. The global public spending on agricultural research and development rose markedly from 26.1 billion dollars in 2000 to 31.7 billion dollars in 2008; however, many challenges still remain in the agricultural sector [2].

Despite the efforts and progress achieved in recent decades in agriculture, growth and crop productivity are still negatively affected by several stress factors. Most crop plants grow in suboptimal environmental conditions, which prevent the plants from expressing their full genetic potential for development and reproduction, and consequently, these abnormal conditions lead to decreased plant productivity [3]. These stresses cause considerable production and economic losses worldwide.

Biological stress is an adverse force or condition that inhibits normal functioning of a plant [4]. These stresses may be biotic or abiotic. Biotic stresses include pathogens (viruses, bacteria, and fungi), insects, herbivores, and rodents. Abiotic stresses comprise cold (chilling and frost), heat (high temperature), salinity (salt), drought (water deficit condition), water excess (flooding), radiation (high-intensity ultra-violet and visible light), chemicals and pollutants (heavy metals, pesticides, and aerosols), oxidative stress (reactive oxygen species, ozone), wind (sand and dust particles in the wind) and soil nutrient deprivation [4, 5]. All of these factors may affect plant development and reproduction at different levels of severity.

Tolerance can be achieved by plant breeding or cultural practices that reduce losses, which is in turn accomplished by understanding the plant's response to its stressors and how they affect individual plants and plant processes [6]. Yield losses by oxidative damages occur because of an imbalance in plant synthesis and quenching. However, attributing this loss to the oxidative damage is difficult taking into account the several processes involved in ROS synthesis; however, stresses and oxidative damage are interlinked and are responsible for the yield losses [7] (Figure 1).





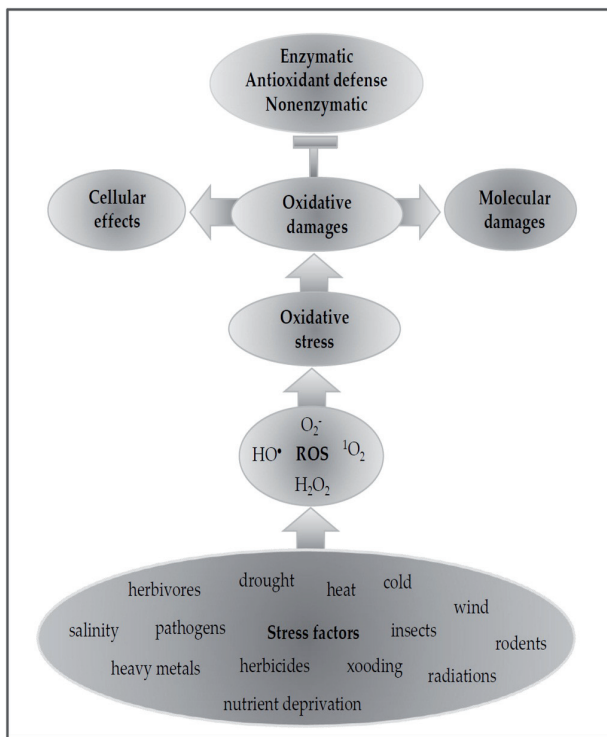
**Figure 1.** Evolution of the number of publications (2000-2014) addressing oxidative damage and yield/production losses. Total number of publications in 2000-2014 is 1,418. Source: ISI Web of Knowledge.

### 3. ROS generation

Environmental stress is directly correlated with increased accumulation of ROS. The equilibrium between production and scavenging of ROS may be disturbed by a number of biotic and abiotic factors, which may increase the intracellular levels of ROS [8]. When the level of ROS is increased and exceeds the defense mechanisms, the cell is in a state of oxidative stress [8, 9, 10, 11]. High concentrations of ROS are highly harmful to organisms, and when the symptoms persist, irreversible damage to the cells occurs, resulting in loss of physiological capacity and eventual cell death. Therefore, defense mechanisms against oxidative damage are activated during stress to regulate toxic levels of ROS [12] (Figure 2).

ROS are a group of free radicals, reactive molecules, and ions derived from oxygen. The most common ROS include singlet oxygen ( $^1\text{O}_2$ ), superoxide radical ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $\text{OH}\cdot$ ). These substances are highly reactive and toxic and can lead to oxidative destruction of the cell [8, 13]. ROS are found in various subcellular compartments such as chloroplasts, mitochondria, and peroxisomes due to the high metabolic activity that normally occurs in these compartments [13]. ROS are generated in chloroplasts via the Mehler reaction, in mitochondria via electron transport, and in peroxisomes via photorespiration.

The glycolate oxidase reaction, fatty acid  $\beta$ -oxidation, enzymatic reactions of flavin oxidases and disproportionation of  $\text{O}_2^{\cdot-}$  radicals are all metabolic processes responsible for the gener-



**Figure 2.** Stress factors, ROS generation, oxidative damage, and antioxidant defense. Several stress factors increased the ROS production, such as  $\text{HO}^\bullet$ ,  $\text{O}_2^\bullet$ ,  $^1\text{O}_2$ , and  $\text{H}_2\text{O}_2$ . The increased ROS levels lead to oxidative stress. Consequently, oxidative damage at the molecular and cellular levels occurs. Defense mechanisms against oxidative stress are activated to neutralize toxic levels of ROS. Singlet oxygen ( $^1\text{O}_2$ ), superoxide radical ( $\text{O}_2^\bullet$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $\text{OH}^\bullet$ ).

ation of  $\text{H}_2\text{O}_2$  in different types of peroxisomes [14]. Cytoplasm, plasma membrane, apoplasts, endoplasmic reticulum, and extracellular matrix are also sources of  $\text{H}_2\text{O}_2$ . In the cytoplasm, the electron transport chain associated with the endoplasmic reticulum is the main source of  $\text{H}_2\text{O}_2$ /ROS [11].  $\text{H}_2\text{O}_2$  generation can also be via enzymatic sources such as plasma-membrane-localized NADPH oxidases, amine oxidases, and cell wall peroxidases [15, 16]. Different organelles and cellular compartments possess potential targets for oxidative damage, as well as mechanisms for eliminating excess ROS. However, the balance between production and elimination of ROS can be severely disturbed by several biotic and abiotic stresses [9, 15]. These disturbances in the ROS equilibrium can lead to a rapid increase in intracellular ROS levels, which can cause significant damage to cell structures [17]. The redox homeostasis is the equilibrium between the production and scavenging of ROS; however, when ROS production overcomes the cellular scavenging capacity, there occurs an unbalancing of the cellular redox

homeostasis resulting in a rapid and transient excess of ROS, known as oxidative stress [11, 12]. Thus, the antioxidant defense imbalance disrupts metabolic activities [18], causing severe oxidative damages to cellular constituents, which can lead to loss of function and even cell death [12].

ROS may affect many cellular functions, for example, they can damage nucleic acids (oxidation of deoxyribose, strand breaks, removal/deletion of nucleotides, modification of bases, and cross-linked protein-DNA), lipids (breaking of the chain and increasing the fluidity and permeability of the membrane), and proteins (site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, alteration of the electric charge, inactivation of enzymes, and increasing the susceptibility of proteins to proteolysis) and can activate programmed cell death [10, 11].

The balance between production and elimination of ROS at the intracellular level must be tightly regulated and/or efficiently metabolized. This is necessary to avoid potential damage caused by ROS to cellular components as well as to maintain growth, metabolism, development, and overall productivity of plants. This equilibrium between the production and detoxification of ROS is sustained by enzymatic and nonenzymatic antioxidants [13, 15].

In plants, the major ROS-scavenging pathway is the ascorbate–glutathione cycle (AsA-GSH) in chloroplasts, cytosol, mitochondria, apoplast, and peroxisomes. This cycle plays a crucial role in controlling the level of ROS in these compartments [15]. The AsA-GSH cycle involves successive oxidation and reduction of ascorbate, glutathione, and NADPH catalyzed by ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [15, 18]. Thereby, the AsA-GSH cycle plays an important role in combating oxidative stress induced by environmental stress. Many components of the antioxidant system of plants are already well characterized into plant models, and disturbances or alterations in this system are an excellent strategy to investigate the different signaling pathways involving ROS.

### 3.1. Nonenzymatic antioxidants

Nonenzymatic antioxidants are found in all cellular compartments. These compounds may act directly in the detoxification of ROS and radicals, or they can reduce substrates for antioxidant enzymes [15]. Nonenzymatic components of the antioxidative defense system include the major cellular redox buffers ascorbate (AsA) and glutathione (GSH) as well as tocopherol, carotenoids, and phenolic compounds [10, 13, 18].

**Ascorbate** is found in organelles of most plant cell types and in the apoplast. AsA is a crucial component of the detoxification of ROS in the aqueous phase due to the ability to donate electrons in enzymatic and nonenzymatic reactions. AsA can directly eliminate  $O_2^{\cdot-}$ ,  $OH^{\cdot}$ , and  $^1O_2$ , and thus reduce  $H_2O_2$  to water via the ascorbate peroxidase reaction [19]. AsA is generally maintained in its reduced state by a set of NAD(P)H-dependent enzymes, including monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase [13, 20, 21]. Moreover, AsA is involved in the regulation of cell division, the progression of G1 to S

phase of the cell cycle and cell elongation, and it participates in multiple functions in photosynthesis [22].

**Glutathione** is oxidized by ROS to form oxidized glutathione (GSSG), which is present in all cellular compartments. Along with its oxidized form, GSSG, GSH maintains the redox balance in cellular compartments. Several studies indicate that GSH is involved in regulating gene expression and the cell cycle due to the properties of the GSH:GSSH pair [15]. The glutathione and AsA antioxidants are abundant and stable and have appropriate redox potential to interact with numerous components and pathways.

**Tocopherols** ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) is a group of lipophilic antioxidants [11]. The  $\alpha$ -tocopherol is the largest scavenger of peroxy radicals in lipid bilayers. The  $\alpha$ -tocopherol present in the membrane of chloroplasts protects them against photooxidative damage [19].

**Phenolic compounds** are abundantly found in plant tissues, such as flavonoids, tannins, hydroxycinnamate esters, and lignin, and possess antioxidant properties [23].

### 3.2. Enzymatic antioxidants

Enzymatic components of the antioxidative defense system comprise several antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), guaiacol peroxidase (POX, EC 1.11.1.7), and peroxiredoxins (Prxs, EC 1.11.1.15), which catalyze ROS degradation, and enzymes of the ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX, EC 1.1.1.1), monodehydroascorbate reductase (MDAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.8.1.7), that regenerate soluble antioxidants [13, 15, 18, 24]. This antioxidant system plays an important role in the maintenance of cell homeostasis and in the antioxidant response in plants.

**Superoxide dismutases** are enzymes that catalyze the dismutation of  $O_2^{\cdot -}$  to  $H_2O_2$ ; therefore, they constitute a frontline in the defense against ROS. These enzymes may be attached to a metal ion (Cu/Zn, Mn, Fe, and Ni); thus, they are classified according to their subcellular location and metal cofactor. SODs are present in many organisms, such as bacteria, yeast, animals, and plants. Plants have multiple genes encoding SODs that can be regulated by development, tissue-specific and environmental signals [10, 25].

**Catalases** are responsible for the removal of  $H_2O_2$  by reducing  $H_2O_2$  to  $2H_2O$ . CATs are largely, but not exclusively, localized to peroxisomes. Plants possess multiple CATs encoded by specific genes, which respond differentially to various stresses that are known to generate ROS [9, 10].

**Ascorbate peroxidases** are enzymes that play a key role in catalyzing the conversion of  $H_2O_2$  into  $H_2O$  and use ascorbate as a specific electron donor. Plants have different APX isoforms that are distributed in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisomes, and the cytosol. The APX genes are differentially modulated by several abiotic stresses in plants [26, 27, 28]. The balance between SODs, CATs, and APXs is crucial for

determining the effective intracellular level of  $O_2^{\cdot -}$  and  $H_2O_2$ , and changes in the balance of these appear to induce compensatory mechanisms [8, 9, 10].

**Glutathione peroxidases** are nonheme thiol peroxidases that catalyze the reduction of  $H_2O_2$  or organic hydroperoxides to water. The GPX proteins have been identified in many life species [29]. In plants, the GPX proteins are localized to mitochondria, chloroplasts, and cytosol.

**Peroxiredoxins** are a family of thiol-specific antioxidant enzymes that are involved in cell defense and protection from oxidative damage. These enzymes are widely distributed in plant cells and are important proteins in chloroplast ROS detoxification [30]. The peroxiredoxins are a group of peroxidases that have reducing activity in their active sites via cysteine residues. These enzymes do not possess a prosthetic group and catalyze the reduction of  $H_2O_2$ , peroxynitrite, and a wide variety of organic hydroperoxides to their corresponding alcohols [31].

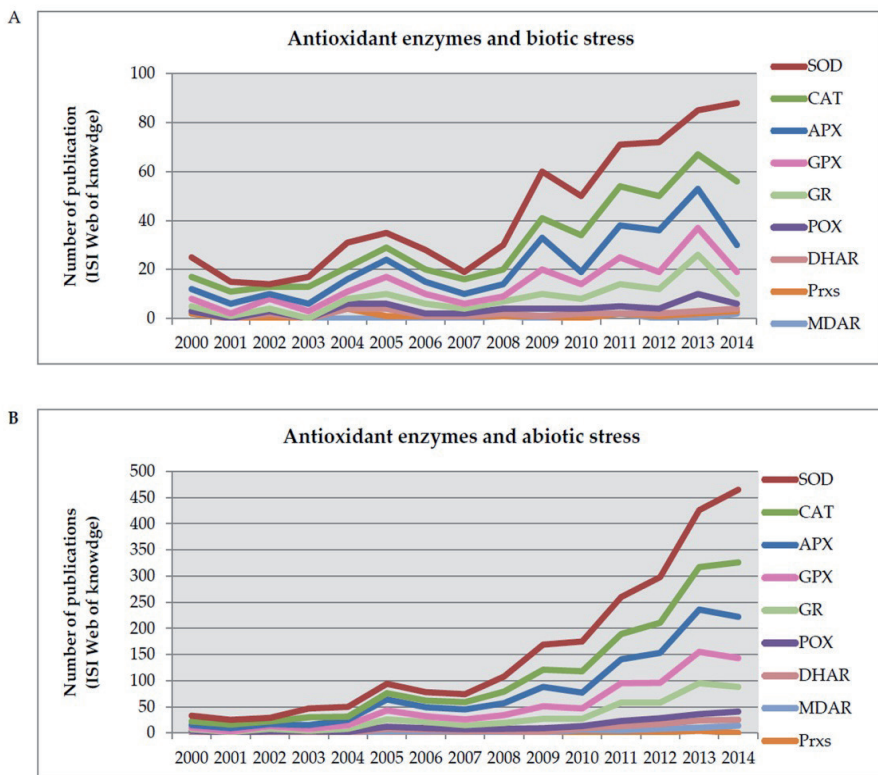
**Guaiacol peroxidases** are involved in  $H_2O_2$  detoxification. The POX proteins are heme-containing enzymes that belong to class III or the “secreted plant peroxidases.” These enzymes are able to undertake a second cyclic reaction, called the hydroxylic reaction, which is distinct from the peroxidative reaction. Due to the use of both cycles, class III peroxidases are known to participate in many different plant processes, from germination to senescence, auxin metabolism, cell wall elongation, and protection against pathogens [32].

**Monodehydroascorbate reductase** is a flavin adenine dinucleotide enzyme that catalyzes the regeneration of AsA from the monodehydroascorbate radical using NAD(P)H as an electron donor. Thereby, MDAR plays an important role in the plant antioxidant system by maintaining the AsA pool [24]. Isoforms of MDAR have been reported to be present in chloroplasts, the cytosol, peroxisomes, and mitochondria [33, 34].

**Dehydroascorbate reductase** is a thiol enzyme that maintains AsA in its reduced form. DHAR catalyzes the reduction of dehydroascorbate to AsA using GSH as a reducing substrate [18, 24]. It is present in various plant tissues, and its modulation activity has been reported in various plant species [35].

**Glutathione reductase** is an NAD(P)H-dependent enzyme. GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH); it is a key enzyme of the AsA-GSH cycle; it protects cells against oxidative damage; and it maintains adequate levels of reduced GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress [20].

The great increasing number of publications addressing APX, SOD, CAT, POX, GPX, Prxs, MDAR, DHAR, and GR enzymes are examples of positive responses to biotic and abiotic stresses by these enzymes. Over the past fourteen years, significant efforts have been made to understand plant antioxidant system mechanisms related to stresses, so the number of publications reporting antioxidant enzymes and biotic and abiotic stresses has increased substantially (Figure 3A and 3B, ISI Web of Knowledge database). These data show the relevance of studying these enzymes assisting in the understanding of its involvement with scavenging of cell toxic products in diverse species and the relation between oxidative stress and biological processes.



**Figure 3.** Evolution of the number of publications addressing antioxidant plant enzymes and biotic and abiotic stresses in the last fourteen years. (A) Data of the antioxidant enzymes and biotic stresses; (B) Data of the antioxidant enzymes and abiotic stresses. SOD (superoxide dismutase), APX (ascorbate peroxidase), CAT (catalase), GPX (glutathione peroxidase), POX (guaiacol peroxidase), Prxs (peroxiredoxins), MDAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase), and GR (glutathione reductase) enzymes. Source: ISI Web of Knowledge.

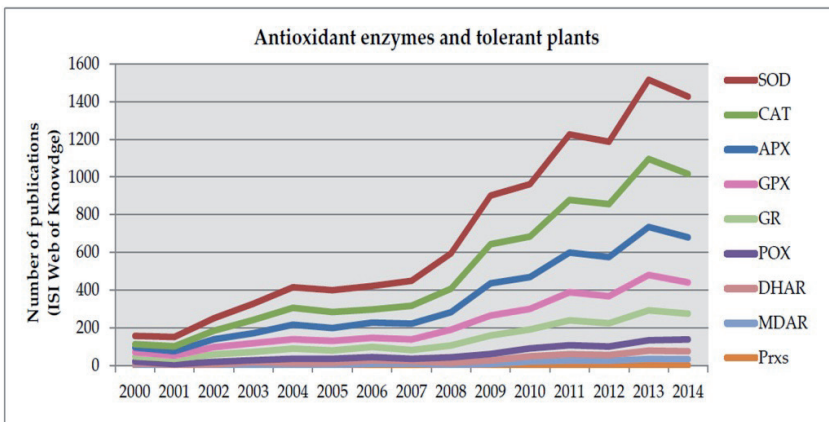
#### 4. Stress conditions and plants tolerant to stress

Stressful conditions are the main factor limiting agricultural productivity because plants do not reach their full genetic potential [4, 17]. Environmental conditions affect growth and development and trigger a series of morphological, physiological, biochemical, and molecular changes in plants. The metabolic pathways of plant organelles are sensitive to changes in environmental conditions [36]. Consequently, all environmental adversities have led to the world's agriculture facing serious challenges to meet demand. The increased consumption, allocation of land for other uses, and use of chemical products with implications for health safety are some examples these challenges [37].

The estimated world population for the year 2050 is nine billion people [2], and, consequently, the food demand will rise again. Therefore, it is necessary to increase the production and quality of food. Currently, the goal of many studies is the understanding of defense/tolerance mechanisms to different stresses in plants and to develop technologies and products that enable the generation of resistant/tolerant and more productive plants. Due to advances in molecular approaches, several crops of economic importance are being produced containing genes that encode stress tolerance using transformational technologies. Thus, several stress signaling and regulatory pathways have been elucidated and better understood.

Knowledge about the oxidative mechanisms in plants may contribute to the development of plants most well adapted to the environment. The maintenance of high antioxidant capacity to remove toxic levels of ROS has been related to increased stress tolerance of crop plants. Several studies show that maintaining a high level of antioxidant enzymes will help a plant to protect itself against oxidative damage by rapidly scavenging the toxic levels of ROS in its cells and restoring redox homeostasis.

Considerable progresses have been achieved in the development of plants tolerant to oxidative stress due to transgenic plants with altered levels of antioxidant genes to improve tolerance and productivity. This fact can be observed in Figure 4, which shows the increasing number of publications addressing antioxidant genes and its relation to tolerant plants in the last fourteen years (Figure 4). It highlights that SOD, CAT, and APX genes are the main antioxidant genes involved in the tolerance of plants to stresses, followed by GPX, GR, POX, DHAR, MDAR, and Prxs, respectively. These studies reflect the importance and advances in comprehension of the antioxidant mechanisms and tolerance to stresses.



**Figure 4.** Evolution of the number of publications addressing antioxidant enzymes and plants tolerant to stresses in the last fourteen years. SOD (superoxide dismutase), APX (ascorbate peroxidase), CAT (catalase), GPX (glutathione peroxidase), POX (guaiacol peroxidase), Prxs (peroxiredoxins), MDAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase), and GR (glutathione reductase) enzymes. Source: ISI Web of Knowledge.



Furthermore, the increased antioxidant activity has been reported to lead to better performance or tolerance response to several stresses. Using transgenic approaches, several species were studied aiming at the improvement of tolerance to stress enhancing antioxidant capacity of antioxidant genes. Table 1 shows some examples of the successful and positive responses obtained with regard to increased tolerance to cold, drought, heat, salt, hydrogen peroxide, methyl viologen, and metals stresses (Table 1). Improved tolerance using antioxidant genes are attributed by high antioxidant activity and more efficient ROS elimination. Plants expressing or overexpressing one or more antioxidant genes have more antioxidant capacity; consequently, plants can more efficiently eliminate excess ROS and protect their cellular components against toxic effects of ROS produced during the exposure to stress. As a consequence, plants suffer less oxidative injury and can tolerate a stress condition more effectively.

Gene	Native specie	Target specie	Stress tolerance	Reference
<i>Ascorbate peroxidase</i>	<i>Brassica campestris</i>	<i>Arabidopsis thaliana</i>	heat	[38]
	<i>Puccinellia tenuiflora</i>	<i>Arabidopsis thaliana</i>	salinity, hydrogen peroxide	[39]
	<i>Jatropha curcas</i>	<i>Nicotiana tabacum</i>	salinity	[40]
	<i>Hordeum vulgare</i>	<i>Arabidopsis thaliana</i>	zinc, cadmium	[41]
<i>Superoxide dismutase</i>	<i>Arachis hypogaea</i>	<i>Nicotiana tabacum</i>	salinity, drought	[42]
	<i>Tamarix androssowii</i>	<i>Populus davidiana</i> x <i>P. bolleana</i>	salinity	[43]
	<i>Pisum sativum</i>	<i>Oryza sativa</i>	drought	[44]
	<i>Oryza sativa</i>	<i>Nicotiana tabacum</i>	salinity, water, PEG-treatment	[45]
<i>Catalase</i>	<i>Brassica oleracea</i>	<i>Arabidopsis thaliana</i>	heat	[46]
	<i>Brassica juncea</i>	<i>Nicotiana tabacum</i>	cadmium	[47]
	<i>Triticum aestivum</i>	<i>Oryza sativa</i>	cold	[48]
<i>Glutathione peroxidase</i>	<i>Triticum aestivum</i>	<i>Arabidopsis thaliana</i>	salinity, hydrogen peroxide	[49]
<i>Peroxioredoxins</i>	<i>Solanum tuberosum</i>	<i>Solanum tuberosum</i>	heat, methyl viologen	[50]
	<i>Festuca arundinacea</i>	<i>Festuca arundinacea</i>	heat, methyl viologen	[51]
	<i>Suaeda salsa</i>	<i>Arabidopsis thaliana</i>	salinity, cold	[52]
<i>Monodehydroascorbate reductase</i>	<i>Malpighia glabra</i>	<i>Nicotiana tabacum</i>	salinity	[53]
	<i>Acanthus ebracteatus</i>	<i>Oryza sativa</i>	salinity	[54]
	<i>Avicennia marina</i>	<i>Nicotiana tabacum</i>	salinity	[55]



Gene	Native specie	Target specie	Stress tolerance	Reference
<i>Dehydroascorbate reductase</i>	<i>Oryza sativa</i>	<i>Oryza sativa</i>	salinity	[56]
<i>Gluthatione reductase</i>	<i>Brassica campestris</i>	<i>Nicotiana tabacum</i>	methyl viologen	[57]
<i>Ascorbate peroxidase/ Superoxide dismutase</i>	<i>Rheum austral/Potentilla astrisanguinea</i>	<i>Arabidopsis thaliana</i>	cold	[58]
	<i>Manihot esculenta</i>	<i>Manihot esculenta</i>	methyl viologen, hydrogen peroxide, cold	[59]
	<i>Solanum tuberosum</i>	<i>Solanum tuberosum</i>	heat, methyl viologen	[60]
<i>Catalase/Superoxide dismutase</i>	<i>Gossypium hirsutum</i>	<i>Gossypium hirsutum</i>	salinity, methyl viologen	[61]

**Table 1.** Some examples of the transgenic plants with potential stress tolerance expressing antioxidant genes

Some antioxidant enzymes such as SOD, CAT, APX, and GPX are better studied (Figure 3 and 4), but in general all enzymes have potential defense antioxidant activity helping in scavenging ROS in different ways, either by dismutation of  $O_2^{\bullet}$  to  $H_2O_2$ , reduction of  $H_2O_2$ , maintenance of the AsA pool, or of the adequate levels of GSH and GSSG, that all together maintain the antioxidant balance. In addition, antioxidant enzymes act in different subcellular compartments, thereby assisting in the ROS detoxification in organelles such as chloroplasts, mitochondria, peroxisomes, and in the cytosol. Besides, ROS-scavenging enzymes in various subcellular compartments might have a synergistic effect to improve stress tolerance in plants [59].

Many attempts aiming to increase the tolerance of plants to environmental stresses using antioxidant genes have been made by researchers. However, due to the great complexity of the antioxidant system and plant stress tolerance, we cannot state that ROS scavenging is the only factor that determines the level of tolerance, because other factors and several genes pathways are involved in the stress tolerance in plants. Furthermore, it must be emphasized that stresses often occur in combination; thus, the relation between ROS signaling mechanisms in different stress responses is very complex [62]. When under the effect of a combination of stresses, the plants respond differently than when experiencing just a unique type of stress [63]. Moreover, this can range depending on the plant species and cultivation area. Complexity of the tolerance mechanisms in plants is also a key factor because sometimes the alteration of one gene in the pathway can influence the expression of others, various genes and pathways being involved [64].

## 5. Conclusions

Plants activate antioxidant defense mechanisms under stresses, which helps in the maintenance of the structural integrity of the cell components and presumably alleviates oxidative

damage. Several antioxidant enzymes contribute to plant defense. The manipulation of ROS-scavenging enzyme systems is a worthwhile approach to produce transgenic plants with enhanced tolerance to a wide range of stress conditions; however, this needs to be further explored as many enzymes and isoforms can be involved, and ROS is only one of the potential parameters of plant tolerance against environmental variations and biotic stresses.

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# Antioxidant Enzyme Activities and Abiotic Stress Tolerance Relationship in Vegetable Crops

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Additional information is available at the end of the chapter

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## Abstract

Climatic changes can cause serious reductions in yield and crop quality. Under the threat of climatic changes, one of the precautions to cope is selection and development of resistant vegetable genotypes to abiotic stresses. Several physiological and biochemical reactions and different tolerance levels can occur according to plant species. When plants are subjected to environmental stresses such as salinity, drought, temperature extremes, herbicide treatment and mineral deficiency, the balance between the production of reactive oxygen species (ROS) and the quenching activity of antioxidants is upset, often resulting in oxidative damage. Since activated oxygen species can disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids, plants possess a number of antioxidant enzymes that protect them from these cytotoxic effects. To control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes for scavenging ROS. The high levels of antioxidative enzyme activities were determined in the tolerant genotypes of tomatoes, eggplant, peppers, cucumbers, melons, squash, beans, okra, etc. to several abiotic stress factors. Both the whole plant and in vitro callus culture experiments gave similar results. Antioxidant enzymes can be useful for screening to determine the tolerant and sensitive plant genotypes against abiotic stresses.

**Keywords:** Drought, salinity, oxidative stress, ROS, chilling

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## 1. Introduction

Stress has a strictly defined physical science definition describing the force per unit area acting upon a material, inducing strain and leading to dimensional change. Biologically, stress has also been defined as the overpowering pressure that affects the normal functions of individual life or the conditions in which plants are prevented from fully expressing their genetic potential for growth, development and reproduction. In the agricultural regard, stress has been

described as a phenomenon that limits crop productivity or destroys biomass. It has become traditional to divide stresses experienced by plants into two major categories: biotic and abiotic stresses. Biotic stresses originate through interactions between organisms, while abiotic stresses are those that depend on the interaction between organisms and the physical environment. Abiotic stresses include potentially adverse effects of salinity, drought, flooding, chilling, metal toxicity, nutrient deficiency, UV exposure, air pollution, etc. [1]. The abiotic stresses represent the factors that most limit the agricultural productivity worldwide. These stresses not only have an impact on current crop species, but they are also significant barriers to the introduction of crop plants into areas that are not currently being used for agriculture [2].

When plants are subjected to environmental stresses such as salinity, drought, temperature extremes, herbicide treatment and mineral deficiency, the balance between the production of reactive oxygen species (ROS) and the quenching activity of antioxidants is upset, often resulting in oxidative damage.

In plants, there are a number of possible ROS sources. These include reactions such as photosynthesis and respiration found in the normal metabolism of plants. This is parallel with the well-known idea that ROS are certain to be one of the products output by aerobic respiration. Pathways that are embellished during abiotic stress also result in ROS production, such as during the photorespiration reaction, where glycolate oxidases in peroxisomes result in superoxidase production. Nonetheless, new sources of ROS have been found recently in plants, such as NADPH oxidases, cell wall-bound peroxidases and amine oxidases. They are involved in ROS production in such processes as cell death and are highly regulated [3]. The ROS are associated with several forms of cellular damage. Since activated oxygen species such as superoxide ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $\cdot OH$ ) can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids, plants possess a number of antioxidant enzymes that protect them from these potential cytotoxic effects [4–7].

Plant species and cultivars within a crop species differ greatly in their response to environmental stress. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage [6–9]. Reports suggest that the extent of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of their antioxidant systems [10].

In general, two classes of nonenzymatic antioxidants are found. They are lipid-soluble membrane-associated antioxidants (e.g.  $\alpha$ -tocopherol and  $\beta$ -carotene) and water-soluble reductants (e.g. glutathione, phenolics and ascorbate). Ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) compose enzymatic antioxidants and they are thought to search for  $H_2O_2$  in chloroplast and mitochondria. Catalase (CAT) and peroxidase (POD) are the other enzymatic antioxidants and are able to remove  $H_2O_2$  and can neutralise or scavenge oxyintermediates and free radicals [11]. Key enzymes involved in the detoxification of ROS are, namely, SOD, CAT, POD, APX and other enzymes implicated in the Halliwell and Asada cycle (ascorbate–glutathione pathway). Under stress conditions, these antioxidants enhance the activity of almost all of these enzymes [11]. Superoxide radicals that emerge as a result of stress in the plant tissues are transformed into hydrogen peroxide ( $H_2O_2$ )

by the SOD enzyme [12, 13]. The accumulation of  $H_2O_2$ , which results from the canalisation reaction of the SOD enzyme and is a powerful oxidant, is prevented by the ascorbate–glutathione cycle. The hydroxyl radical (OH), which is very reactive and the most toxic oxide, can react with all macromolecules without discrimination. SOD and CAT, by combining their actions, can prevent or decrease the formation of this oxide. Even though the particular scavengers are not fully known of the single radical oxygen or the hydroxyl radical, it is thought that SOD functions in removal via chemical reaction [11]. In the defence against intracellular antioxidants, SOD and GSH work together and SOD prevents the radical-mediated chain oxidation of GSH, thus helping GSH in its role as a free radical scavenger physiologically, without the accompaniment of oxidative stress [11, 14]. It was observed that with continued stress conditions, SOD enzyme activity, which acts by decreasing the oxidative oxygen species derived from stress, continued to increase. Even though the linearity of increased stress duration and the increase of SOD activity is concurrent, it was shown that genotypes with more tolerance are superior in this area. The CAT enzyme changes oxidative stress-induced reactive oxygen derivatives, like  $H_2O_2$ , into water and molecular oxygen [15]. CAT, found mostly in glyoxysomes of lipid-storing tissues in plants, contains a tetrameric haeme that catalyses the conversion of hydrogen peroxide, produced from the  $\beta$ -oxidation of fatty acids, into water and oxygen [11, 16]. The GR and APX enzymes, which are a part of the defence mechanism of tolerant genotypes against salt, drought and chilling stress, are generally effective in the reduction of hydrogen peroxide to water in chloroplasts and mitochondria, thereby detoxifying them [17, 18]. APX is one of the most important antioxidant enzymes of plants that detoxify  $H_2O_2$  by using ascorbate for reduction. Different isoforms of APX are active in chloroplasts, cytosol and microsomes [11]. In the ascorbate–glutathione cycle, APX reduces  $H_2O_2$  into water by oxidising ascorbate into monodehydroascorbate (MDHA), which is then converted into ascorbate via the MDHA reductase enzyme; thus, two MDHA molecules are changed into MDHA and dehydroascorbate (DHA) as a non-enzymatic side product in unequal amounts. Subsequently, the reduction of DHA occurs and ascorbate is produced by the action of dehydroascorbate reductase (DHAR) and GR. DHAR can then convert GSH into glutathione disulphide (GSSG) which then is reduced back into GSH by GR [18, 19]. Due to APX activity resulting in the need for regenerating ascorbic acid, it is thought that concurrently an increase in various other components of the antioxidative defence system is needed so that the protective mechanisms of plants can increase as necessary [11]. POD, CAT and APX appear to play an essential protective role in the scavenging process when coordinated with SOD activity. They scavenge  $H_2O_2$  generated primarily through SOD action [11, 20].

The research indicates that APX, CAT, GR and SOD enzyme activities in large variation among cotton varieties [21], tomato [22–24] and melon genotypes [25] in their response to salinity have been observed.

The tolerance of plants to stress has been widely shown to vary with physiological growth stage, developmental phase and size of plants. There is also growing evidence of multiple tolerances to stress in plants with plants showing tolerance to more than one stress. Genetic variability within a species is a valuable tool for screening and breeding for higher stress tolerance.

## 2. Salinity stress

Salinity is one of the most important abiotic stresses that cause reduction in plant growth, development and productivity worldwide in arid and semi-arid regions, where soil salt content is naturally high and precipitation can be insufficient for leaching. The FAO estimates that 34 million hectares of irrigated land are salt-affected worldwide, and an additional 60–80 million hectares are affected by waterlogging and related salinity [26].

Salt stress changes the morphological, physiological and biochemical responses of plants. There is evidence that high salt concentrations cause an imbalance in cellular ions, resulting in ion toxicity and osmotic stress, leading to the generation of ROS, which cause damage to DNA, lipids and proteins. At the same time, ROS cause chlorophyll degradation and membrane lipid peroxidation, decreasing the membrane fluidity and selectivity. To prevent the negative effects of ROS, plants have developed various antioxidant enzyme systems including non-enzymatic antioxidants (e.g. ascorbic acid, glutathione and carotenoids) and antioxidative enzymes (e.g. GR, SOD and APX). While CAT and peroxidases detoxify the toxic hydrogen peroxide, superoxide is broken down into water and oxygen by catalyses from the SOD enzyme. APX reduces  $H_2O_2$  using ascorbate as an electron donor in the ascorbate–glutathione cycle. Oxidised ascorbate is then reduced by GSH generated from GSSG catalysed by GR at the expense of NADPH. Previous studies showed that the level of antioxidative enzymes increases when plants are exposed to oxidative stress including salinity [27–29].

Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage. Genetic variability within a species is a valuable tool for screening and breeding for higher salt tolerance. Some authors have reported large variation among cotton varieties and tomato genotypes in their response to salinity. The response of plants to salt stress is variable and dependent on various factors, particularly plant genotype, extent of stress, age of plant and stage of plant growth when stress is experienced. Plants manifest themselves with various adaptive mechanisms (morphological, biochemical, enzymatic and physiological) to survive under stressed conditions. The extent of these mechanisms' adaptability is unique with every plant genotype [30].

In one recent study, the relationship between antioxidant enzymes and salt tolerance in the leaves of eggplant seedlings of two salt-tolerant varieties (Burdur Bucak and Mardin Kızıltepe) and two salt-sensitive genotypes (Giresun and Artvin Hopa) was examined. In salt-tolerant eggplants, APX, CAT, GR and SOD activities increased significantly when the seedlings were grown in the hydroponic system containing 150 mM NaCl [31]. It was observed, in the results of pumpkin salt tolerance studies, that in the salt-sensitive genotypes (CU-7 and A-24) less of the aforementioned enzymes showed activity as opposed to the increased activity seen in higher salinity levels and in the salt-tolerant genotypes (Iskenderun-4 and AB-44). It was also shown that these enzymes played a role in salinity tolerance of melons [32, 33], green bean [6] and soybean [8].

The salinity experiment in okra genotypes shows that NaCl-induced stress caused decreases in plant biomass, green pigments, photosynthetic activity, stomatal conduction, transpiration rate, number of stomata and stomatal size and resulted in alterations in enzymatic activities

(SOD, POD and CAT) and osmolyte accumulation (proline, glycine betaine, total free amino acids and total soluble sugars). The increase in Na and Cl and lipid peroxidation under saline conditions is the indication of ion toxicity and oxidative damage. However, the oxidative damage is controlled by a defensive system comprising various antioxidants, such as SOD, POD and CAT. The results depicted that salt-tolerant and salt-sensitive genotypes exposed to NaCl stress showed the highest activities of SOD, POD and CAT, both in root and leaf tissues of okra genotypes [30].

According to Yadav et al. [34], plants with high levels of antioxidants have been reported to have greater resistance to this oxidative damage and an increase in the activity of antioxidative enzymes in plants under salt stress. It was observed that increased levels of antioxidants in plants resulted in them having a stronger resistance against the oxidative damage, while plants under salt stress were shown to have the activity of antioxidative enzymes to become greater and that a correlation was seen between tolerance against salt levels and the amount of enzymes. It was also observed that in pea cultures, antioxidant enzyme activity increased when in saline environments, but SOD was unaffected in cucumber. The variations in these observations maybe due to the fact that the effects of salinity depend on a number of factors, for example, salt type, their concentration, plant genotype, growth stage and/or environmental conditions. The mechanism by which salinity affects the antioxidant responses is not yet clear. However, proposed that it might be via the change in membrane integrity caused by high Na<sup>+</sup> to Ca<sup>2+</sup> ratio [34].

Salinity inhibition of plant growth is the result of low osmotic potential of soil (water stress), nutritional imbalance, specific ion effect (salt stress), or a combination of these factors. Grafted plants also exhibit phenotypic variations from scion and rootstock plants in terms of salinity tolerance, and grafting onto salt-tolerant rootstocks capable of inducing salt tolerance in the grafted shoots has been an effective method for improvement of salt tolerance in agricultural practices [35]. In parallel, *Solanum lycopersicum* L. ('Elazığ') grafted on *Nicotiana tabacum* L. ('Samsun') and *Nicotiana rustica* L. ('Hasankeyf'), namely "Tomacco" plant (patent no. TR-2008-05391-B), to 10-d high NaCl irrigation. Physical development, chlorophylls a and b, total chlorophyll, total carotenoid and anthocyanin levels were evaluated [36]. During an increase in osmotic stress levels, plants utilise various antioxidant enzymes and increase their activity; thus these enzymes play vital roles in ROS removal. SOD, which is an important scavenger, is used for producing water and oxygen by catalysing O<sub>2</sub><sup>•-</sup>; afterwards the ROS hydrogen peroxide is catalysed by POD and CAT to produce water and oxygen as well [37]. It was reported by Azevedo Neto et al. [38] that antioxidative enzymes (e.g. POD, SOD, GR, CAT and APX) in addition to low molecular mass antioxidants make up the complicated antioxidative defence system seen in plants. It was observed that grafted seedlings when under stress by over the normal amount of Ca(NO<sub>3</sub>)<sub>2</sub>, contents of hydrogen peroxide and malondialdehyde (MDA), had a much lower percentage of electrolyte leakage and O<sub>2</sub> production rate when compared to those of non-grafted seedlings from which can be deduced that there was less damage to the membrane of grafted seedlings than that of non-grafted seedlings when under excess Ca(NO<sub>3</sub>)<sub>2</sub> stress. It was also observed that in grafted seedlings, free radical scavenging systems had a significant job when battling salinity stress, thus resulting in salinity tolerance [38]. In parallel with these results, Wei et al. [39] determined that due to the efficient

scavenging system of free radicals in addition to the mechanisms that utilise antioxidative enzymes and polyamines for protection, eggplant seedlings that were grafted had a greater resistance against stress of over the normal amount of  $\text{Ca}(\text{NO}_3)_2$  than when compared to non-grafted eggplant seedlings. For rootstock, they used the eggplant cultivar, Swartz cv. Torvum Vigor, which were tolerant to salinity [39]. Plant tissue culturing has been important in many fields including both agriculturally and commercially and has a significant role in the production of ornamental plants. It has also been invariably useful in manipulating plants for enhanced agronomic performances. In vitro culturing of plant cells provides a means for conducting many studies and improvement in scientific research, including helping to study plant physiology and genomics and their processes and enhancing genetic variability by providing the possibility to help in the breeding of improved cultivars. Thus, it has attracted a large amount of interest in later years. Regenerated plants are expected to have the same genotype as the donor plant; however, in some cases, somaclonal variants have been found among regenerated plants [40]. According to Kusvuran et al. [18], the responses to salt and drought stress of four pumpkin varieties (A-24, CU-7, Iskenderun-4 and AB-44) were investigated under in vitro culture conditions. In this study, it was observed that the tolerant genotypes had less damage on their cell walls (by lipid peroxidation) than that of the sensitive genotypes. While, it was observed that salinity stress had greater effect on the pumpkin genotype than stress by drought. The results of this study suggest that antioxidative defence mechanisms were effective in the pumpkin callus tissues during salt and drought stress. Thus, the increased activity of antioxidative enzyme activity as well as the lower increasing MDA content in the salt- and drought-adapted cells compared with the unadapted cells may contribute to salt and drought tolerance. The results obtained with the callus tissues are in agreement with those observed in studies using seedlings. The results demonstrate the selection of tolerant genotypes for oxidative stress such as drought; salinity could be used for in vitro methods [18]. Similarly, other research on squash, eggplant and melon indicated that SOD, CAT and APX enzyme activities in salt-tolerant genotypes are higher compared to salt-susceptible genotypes in both seedling and callus tissues [41–43].

Antioxidant enzymes such as SOD, POX and CAT are known to substantially reduce the levels of superoxide and hydrogen peroxide in plants. It is one of the most important enzymes used against oxidative stress in the plant defence system, and it occurs ubiquitously in every cell of all types of plants. The most common isoforms of SOD known in the literature are copper–zinc containing SOD (Cu/Zn-SOD), manganese containing SOD (Mn-SOD), iron containing SOD (Fe-SOD) and nickel containing SOD (Ni-SOD) [44]. Moharramnejad and Valizadeh [45] observed the three isoforms for SOD and POX and one isoform for CAT. Authors showed that in salt stress, the main activities of SOD, POX and CAT isozymes are significantly higher than normal conditions in red bean (*Phaseolus vulgaris* L.).

### 3. Drought stress

Drought stress is one of the most serious abiotic stresses that cause a reduction in plant growth, development and yield in many parts of the world [46–48]. However, plants have developed

different morphological, physiological and biochemical mechanisms to withstand drought stress [49–51]. It is of considerable value to understand the reactions and responses of plants in drought environments as it is also a major part in making crops more tolerant towards stress. It is important to note that responses of plants towards water stress are seen to be considerably different at organisational levels according to the intensity and time they spend under the stressful environment as well as the period of growth they are in and their species [52]. When water becomes limited, the plant generally experiences stomatal closure in an effort to prevent further water loss, limiting the carbon dioxide available for fixation by photosynthesis and reducing NADP<sup>+</sup> regeneration by the Calvin cycle [53]. These converse conditions increase ROS, such as hydrogen peroxide, superoxide, singlet oxygen and hydroxyl radicals [6, 54]. These ROS attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturing and DNA mutation. Plants possess several antioxidant enzyme systems that protect their cells from the negative effects of ROS. The role of antioxidant enzymes (APX, CAT, SOD and GR) as the components of the main tolerance mechanism is developed in response to different stress conditions. Many reports suggest that the extent of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of their antioxidant systems and the relationship between enhanced or constitutive antioxidant enzyme activities and an increased resistance to drought stress [55–57].

In one study [58], the effects of drought stress on plant growth, relative water content (RWC), ion concentration and activities of the antioxidant enzymes, APX and GR, in eight okra genotypes were investigated. Drought-resistant genotypes exhibit a better protection mechanism against oxidative damage by maintaining a higher inherited and induced activity of antioxidant enzymes than the sensitive genotypes. Previously, 31 different melon genotypes grown under salt and drought conditions were classified according to some growth parameters (i.e. shoot length, plant leaf area, leaf number, fresh and dry weight, leaf water content, ion accumulation and membrane injury index), as well as some antioxidant enzymes activities *in vivo*. At the end of the study, two salt- and drought-tolerant genotypes and two sensitive genotypes were selected according to the growth parameters measured. The aim of this study was to determine the activities of antioxidative stress enzymes in some salt- and drought-sensitive or salt- and drought-tolerant melon varieties grown in *in vitro* culture under salt and drought stress conditions. Another aim of this study was to determine whether *in vitro* callus culture can be used as a screening method for salt and drought stresses in a melon screening study. In contrast with the control, in all the different 8-day salinity and drought stress, it was discovered that growth prevention occurred in callus tissues within media containing 100 mM NaCl or 15% (w/v) PEG-6000 (polyethylene glycol). It was observed in the study from the MDA measurements, from which the amount of lipid peroxidation of the cell wall can be deduced, that the amount of damage to the cell wall (by lipid peroxidation) was more in the sensitive genotypes than that of the tolerant genotypes. The antioxidant enzyme (APX, CAT, SOD and GR) activities were investigated in the callus tissues of four melon genotypes under salt and drought stress. This study suggested that antioxidative defence mechanisms were effective in the melon callus tissues during salt and drought stress. The results demonstrate the selection of tolerant genotypes for oxidative stress such as drought; salinity could be used for *in vitro* methods [59]. Drought tolerance of tomato genotypes [60] was investigated and found



biochemical changes (drought stress index, MDA content and antioxidant enzyme activities) that occur as a result of stress in plants were investigated. In salt-tolerant varieties, T-1 and T-2, the decrease of ions occurred at lower levels under drought conditions. APX, CAT, SOD and GR enzyme activities have increased in drought stress conditions. The four varieties showed an increase in MDA content under drought conditions, especially in the sensitive genotypes. The results indicate that the tomato seedlings respond with enzymatic defence systems against drought-induced oxidative stress.

Water stress tolerance is seen in all plant species but its extent varies from species to species. Effects of different PEG concentrations with drought stress on the activity of antioxidant enzymes, CAT and APX, were investigated in two melon genotypes. Drought tolerant (CU-196) and drought sensitive (CU-3) were grown in hydroponic conditions. Recently, PEG has being used as osmotic pressure inducer in drought physiology studies. In the study, 15, 30 and 45 mM PEG-6000 doses ( $-0.15$  MPa,  $-0.52$  MPa and  $-1.50$  MPa, respectively) were compared to CAT and APX antioxidant enzymes in tolerant and sensitive melon genotypes. At the end of the study, CAT and APX enzyme activities significantly increased in CU-196 than CU-3 [61]. In the other study was conducted for determination of tolerance levels to drought of melon genotypes (Midyat, Şemame, Yuva and Ananas) that have determined the levels of tolerance to salt stress. In this study, three different irrigation methods have been applied to plants. (S0: control-plant-available water, 40% is consumed for irrigation, S1: plant-available water, 90% is consumed for irrigation, S2: during the period of 3–4 leaves of plants completely cut off from the irrigation). Morphological and biochemical changes that occur as a result of stress in plants were investigated. Drought stress applied to the visual scale evaluation of melon genotypes in terms of Midyat and Şemame melons had values close to controls. However, Yuva and Ananas genotypes were found to be more pronounced losses caused by drought. Under drought stress, for Midyat and Şemame genotypes that are tolerant to salt stress, plant fresh and dry weight, the values shown in chlorophyll were closer to control values. At the same time, SOD, CAT, GR and APX enzyme activities have increased in drought stress conditions. However, the susceptible varieties (Yuva and Ananas) compared to the control plants in terms of the parameters studied enzyme activities decreased to varying degrees. In general, it also drought-tolerant melon genotypes found to be tolerant groups, respectively. In particular, these melon varieties have enhanced levels of antioxidant enzyme activities by activating the tolerant concluded [62]. Kiran et al. [60] investigated that determination of tolerance levels to drought of tomato genotypes (TR-68516, Rio Grande, TR-63233, TR-63233 and H-2274). Authors indicated that SOD, CAT, GR and APX enzyme activities have increased in drought stress conditions. On the other hand, in the susceptible varieties (TR-63233 and H-2274) compared to the control plants in terms of the parameters studied enzyme activities decreased to varying degrees [60].

Smirnoff [63] indicated that oxidative damage is also manifest in effects on to proteins and nucleic acids, although these are rarely measured and can be affected by other factors. Oxidation of amino acid residues can be followed by the loss of catalytic activity and denaturation. The damaged proteins may be more susceptible to proteolytic degradation. DNA repairing enzymes may also be induced as a result of oxidative damage to DNA. The various



repair and protection systems found in plants decrease the amount of open oxidative damage and consist of two groups. One of the groups includes CAT, ascorbate, PODs and SOD where in these systems react with oxygen forms that are active and keep them at a minimum level. The second group consists of GR, mono and DHARs, glutathione (GSH) and ascorbate, which are involved in the regeneration of antioxidants that are oxidised. For the purpose of maintaining the superoxide concentration at a minimum, and thus decreasing the production of hydroxyl radicals by the Haber–Weiss reaction of which the catalysis are done by metals, superoxide is converted into hydrogen peroxide by the SOD enzyme catalysis from the first group. Three types of SOD occur in plants: Cu/Zn-SOD, Mn-SOD and Fe-SOD. The latter two have similar amino acid sequences. SOD isoforms occur in most of the subcellular compartments (hydrogen peroxide is broken down to water by CAT which is located in peroxisomes and glyoxysomes). In the chloroplast, this function is fulfilled by ascorbate that also has a cytosolic isoform. In plants, a large amount of the PODs can have major roles other than antioxidants, while ascorbate, with both superoxide and singlet oxygen, has the ability to react non-enzymatically too. In the second group, GSH and ascorbate are the key players in the reactions involved in antioxidants becoming regenerated. In the reaction involving APX, the MDHA radical is the major product and goes on to react with NAD(P)H-dependant monodehydroascorbate reductase (MDHAR) to get reduced to ascorbate. Or, ascorbate and dehydroascorbate (DHA) can be produced by the non-enzymatic reaction between two molecules of MDHA, after which ascorbate is produced by the reduction of DHA by GSH and where the enzyme DHAR catalyses the reaction, with the second product as oxidised GSH (GSSG). An NADP-dependant GR can then reduce the oxidised GSH into GSH. In the chloroplast, these reactions, sometimes known as the Halliwell–Asada cycle, result in the catalysis of the light-dependant reduction reaction from hydrogen peroxide into water by the action of the reductant (NADPH) that is produced by photosynthesis. The key players of this cycle ascorbate and GSH, along with their isoforms (GR, DHAR and MDHAR), are found in large amounts in the chloroplast in addition to other subcellular compartments. The oxidation of GSH pools, which are also essential in keeping sulphhydryl groups of enzymes in reduced forms, could result in enzymes that rely upon these reduced SH groups being inactivated [63].

Yasar et al. [64] investigated 38 genotypes of different pumpkin species for the relationship between the drought tolerance capacity and antioxidant enzyme activity. As a result, it was observed that the enzyme activities are extremely vital in the drought tolerance of the pumpkin genotypes, such as under dry conditions, the drought-tolerant pumpkin genotypes use antioxidative enzymes more actively compared to the drought susceptible genotypes. The genotypes exposed to drought stress had relatively inferior SOD enzyme activity compared to their controls. However, the CAT enzyme activities of these genotypes were found to be increased. Alternatively, the opposite situation was also observed; if the CAT enzyme activities were decreased compared to the controls, the SOD enzyme activities were observed to be increased compared to the genotypes in control group. However, such a relationship was not established for the APX enzyme activities [64].

Drought tolerance in black pepper is attained through osmotic adjustment and better ROS scavenging machinery, functioning through different antioxidant enzymes. The activities of antioxidant enzymes such as SOD and POD become higher during stress in tolerant variety [65].

#### 4. Chilling stress

Low-temperature, or chilling, stress (damage caused by low, but above-freezing temperatures) has been recognised as a unique environmental impact on crop plant physiology [66]. The damage resulting from the symptoms of the chilling stress includes a decrease in growth and yield of the plant. These symptoms consist of the prevention of metabolic processes, rise in the permeability and seepage through the cell membrane due to alterations in the order of the molecules or in the physical form, wilting and chlorosis [67]. Prasad et al. [68] has suggested that mitochondria are critical organelles in the metabolic production of energy in the cell. The competence and the stability of mitochondria are very important for the seedlings to survive low-temperature stress, especially during early seedling growth. Low temperature induces oxidative stress in the cell [69]. Under aerobic conditions, superoxide radicals and  $H_2O_2$  are found to be normal metabolites of plant cells [70, 71] as well as animal cells [72, 73] and are kept at low, steady-state levels by the action of antioxidant enzymes such as SOD, CAT, GSH POD and APX located in the organelles and cytosol [74–76].

Active oxygen species (AOS) has been proposed to be responsible for cold-induced injury because they are produced at higher concentration during cold stress and may initiate degradative reactions, causing lipid peroxidation, membrane deterioration, protein degradation and chlorophyll quenching. An efficient antioxidant activity is essential in order to maintain the concentration of AOS at relatively low levels [67]. On the other hand, the damage that occurs during chilling stress accompanying illumination was thought to be mediated by an oxygen radical. The defence mechanisms of the cell against oxidative stress involve antioxidants that can be found in many plant organs in large amounts to perform vital biological functions. These include the enzyme systems CAT, SOD and numerous PODs, e.g. APX and guaiacol peroxidases (POX) [69]. During photosynthesis, superoxide and hydrogen peroxide are produced as side products and need to be removed. This is achieved by the SOD enzyme and the enzymes GSH and ascorbate from the ascorbate–glutathione cycle. It was shown by Aroca et al. [77] that due to ROS being produced under chilled environments during light-induced photo-oxidation, the major damage from chilling stress occurs during this time. The reason for ROS production under these circumstances is because of the slowing down of the enzymes involved in the Calvin–Benson cycle, thus resulting in the limitation of the  $NADP^+$  supplements receiving the electrons from the electron transport chain and inducing oxygen to absorb more energy than needed. To decrease photo-oxidation under chilling, there are three important mechanisms. The first one involves avoiding production of ROS by diminishing electron transport chain; the second one involves scattering surplus energy in the form of heat via violaxanthin de-epoxidation, and the third one involves scavenging ROS produced by antioxidant compounds and enzymes. Additionally, the water-water cycle in the chloroplast, where electrons flow in photosystem II from water to photosystem I to reduce oxygen without

O<sub>2</sub> levels having a net change, is said to be an active mechanism that can disperse energy from over excitation when there is an environmental stress. SOD, GR and APX are also some of the antioxidant enzymes involved in the function of this cycle [77]. The enzyme SOD is located in the cytoplasm, chloroplast, mitochondrion and peroxisome and acts as the first line of defence mechanism against ROS by dismutating O<sub>2</sub> into H<sub>2</sub>O<sub>2</sub> [78]. Furthermore, the dismutation of superoxide radicals into H<sub>2</sub>O<sub>2</sub> and oxygen is an important step in protecting the cell, and in that conversion, SOD is considered a key enzyme [69]. CAT also played a significant role in chilling tolerance and is especially important for removal of H<sub>2</sub>O<sub>2</sub> in C3 plants. Exposure to low temperature may increase the amount of AOS not only in cold-sensitive but also in cold-tolerant plants. There was a correlation between the reduction in CAT activity and H<sub>2</sub>O<sub>2</sub> accumulation [79].

Oxidative free radicals can be highly reactive towards cell components, and therefore, the ability of the cell to remove these undesirable species might be viewed as an important feature in improved resistance to chilling stress. The increases in the activities of CAT3 provide evidence for the increased production of superoxide and H<sub>2</sub>O<sub>2</sub> in mitochondria of maize seedlings. Increases in superoxide and H<sub>2</sub>O<sub>2</sub> can be expected in cases in which there is either high O<sub>2</sub> uptake or decreased ability of the electron transport pathway, which increases potential for higher electron leakage to O<sub>2</sub> for subsequent production of superoxide and H<sub>2</sub>O<sub>2</sub> [68].

One major antioxidant that plays a role in the detoxification of ROS and plant protection against oxidative damage is glutathione. There are two versions in which glutathione can exist in which are the oxidised disulphide version (GSSG) and the reduced version (GSH). The function of glutathione as an antioxidant is mainly assigned to its reduced (GSH) version as this form is oxidised to form the oxidised (GSSG) version during its function as an antioxidant. Therefore, keeping the concentration of reduced glutathione, from the ratio GSH/GSSG, high is important for plants. The production of GSH can occur both in cytosol and the chloroplast in the leaves of the plant. Furthermore, in the ascorbate–glutathione cycle, GR catalyses GSSG reduction into GSH via donation of electrons from NADPH molecules. ROS detoxification in the chloroplast is known to be mostly carried out by the ascorbate–glutathione cycle, which is thus accepted as the main pathway in this process. In this cycle, ascorbate is also considered to be a major antioxidant in addition to GSH [80]. According to Prasad [81], rapidity with which GR enzyme was induced during the early stages of acclimation and remained induced during chilling and recovery clearly suggests that acclimation uniquely induces the antioxidant defence mechanism that is necessary for protecting the seedling from oxidative stress injury.

Chilling-sensitive pepper cultivars were investigated for SOD, CAT and POD enzyme activity under chilling stress condition. The results showed that the activity of CAT decreased, and both SOD and POD activities raised in two cultivars. However, permeability of plasma membrane was positively related to MDA content, SOD and POD activity and also negatively related to CAT activity variation. As low temperature treating was extended, permeability of plasma membrane, MDA content and POD activity increased and SOD and CAT activity decreased in two cultivars: Xiza No. 7 (less chilling-sensitive cv.) could maintain a higher protective enzyme activity, and permeability of plasma membrane and MDA content were low; Hajiao No. 1 (chilling-sensitive cv.) was quite the contrary [82].

Anderson et al. [83] indicated that the response of ascorbate and glutathione in mesocotyls to acclimation and chilling was generally the same as that in the coleoptile + leaf, although the increase in the total glutathione pools in response to acclimation was not as extensive. Induction of other antioxidants in the mesocotyl may reduce the need for GSH synthesis. In the roots, there was no effect of acclimation or chilling on any of the antioxidants tested. However, the fact that  $H_2O_2$  levels in the roots were not greatly increased by chilling suggests that a modification of antioxidants was unnecessary to prevent oxidative stress [83]. Likewise, Lee and Lee [84] established that APX is also an important antioxidant enzyme in scavenging or utilising  $H_2O_2$ . Total APX activity increased when chilling stress occurred in the leaves of the cucumbers and seemed to be because of favoured induction of the isozymes APX-5 and APX-4. However, 24 h after the stress, the increase seen in APX activity was because of the favoured expression of the isoform APX-3 [84]. Chilling stress causes many physiological and biochemical changes. Kang and Saltveit [85] investigated that chilling tolerance in cucumber seedling radicals. Chilling seedlings with radicles 20-mm long for 48 h at 2.5°C inhibited subsequent growth by 36%, while it reduced the growth of 70-mm-long radicles by 63%. APX activity was higher in 20-mm-long radicals before chilling than in 70-mm-long radicles. It appears that higher APX, CAT and DPPH (the stable free radical 1,1-diphenyl-2-picrylhydrazyl)-radical scavenging activities, and sustained APX activity during chilled and during subsequent growth at 25°C following chilling in 20-mm-long radicals corresponds with higher chilling tolerance. The activities of APX, CAT and DPPH appear to be positively correlated with chilling tolerance [85].

A study on the effects of chilling stress on two salt- and drought-tolerant and two sensitive pumpkin genotypes in callus culture [18] found that the tolerant genotypes showed lower increase in lipid peroxidation and a greater increase in APX, CAT and GR than the tolerant genotypes under stress conditions. It was observed that even though the increase of glucose, proline and fructose concentration went up with prolonging of the chilling effect in all the pumpkin genotypes, a much more significant increase was observed in the tolerant genotype than that of the sensitive genotype. Thus it can be concluded from these results that in pumpkin genotypes, chilling stress results in an increase in the peroxidation of lipids and in oxidative stress, due to reactive oxygen radical production. Song et al. [86] in their study observed that chilling stress, SOD and CAT activities decreased in some extent in both cultivars, in comparison to control in tomato cultivars. Compared to control, chilling stress resulted in significantly higher POD activity in cv. Mawa on day 6, whereas no significant changes of POD activity caused by chilling stress were observed in cv. Moneymaker at all time points tested. On the other hands, APX activities were increased in the two cultivars under chilling stress. GR activities increased in cv. Mawa after chilling stress, but almost no change was observed in cv. Moneymaker [86].

## 5. Nutrient deficiency and toxicity of heavy metal

The micronutrients essential for the normal growth and development of plants, as it is known to be required in several metabolic processes [87]. Deficiency of nutrients such as Zn, Mn, Cu,

Fe, Mg, B and K can modify the activities of several antioxidative enzymes [88]. Kosesakal and Unal [89] indicated that Zinc (Zn) is one of the essential micronutrients playing a significant role in many vital metabolic processes. Zinc deficiency is a major global problem hindering plant cultivation, and this problem is especially exacerbated in acidic calcareous soils, which is the most common soil type in arid and semi-arid regions of the world. It is known that magnesium deficiency results in the decrease of chlorophyll amounts in beans. It was observed by Welkie et al. [90] that in peppers, the amounts of chlorophyll and iron in leaves were directly proportional, while zinc was also shown to be beneficial in the synthesis of carotenoids and chlorophyll, thus being proved to be essential in the photosynthetic process in plants. Iron (Fe) is a cofactor of many antioxidant enzymes and could act as a pro-oxidant factor because free or loosely bound it catalyses free radical generation in the presence of reductants and peroxides through the Fenton reaction. The growth of sunflower plants under iron deficiency conditions affects POD isoforms differently, inducing a preferential reduction in activity of those isoforms involved in the detoxification processes [91].

Metal toxicities have received widespread attention as large amounts are released into the environment and affect living organisms. Heavy metal intoxication, especially by lead, cadmium, arsenic and mercury, constitutes serious threat to human health [92, 93]. Although information focussed on the relationship between heavy metals and oxidative stress in plants has been available in recent years, it is still difficult to draw a general conclusion about critical toxic metal concentrations in soils [94]. Heavy metals cause oxidative damage to plants, either directly or indirectly through AOS formations which are extremely toxic to living cells. Redox metals such as Cu or Fe appear to act directly on the production of AOS. Copper is among the major heavy metal contaminants in the environment with various anthropogenic and natural sources. Human health risk from heavy metal bioaccumulation in vegetables has been a subject of growing concern in recent years. Excess Cu inhibits plant growth and seed germination, induces chlorophyll degradation and interferes with photosystem activity. At the molecular level, Cu ions generate ROS. These reactive radicals cause oxidative damage of lipids, proteins and nucleic acids. Cu ions also are responsible for alterations of membrane integrity in plant cells. Cu-mediated membrane lipid peroxidation causes membrane damage, thus changing membrane permeability and leading to electrolyte leakage. Plants have evolved several antioxidant defence mechanisms to protect themselves from oxidative damage [95].

Zn is the second most abundant transition metal after iron (Fe) and is involved in various biological processes in organisms. Due to this, the results of the presence of Zinc were investigated, including zinc deficiency, hyperaccumulation and its protective role in plants. However, it is not clear what the implications of zinc stress are on antioxidant responses and the uptake of nutrition, though it is known that excess of zinc is not beneficial and can result in negative symptoms in plants. The symptoms that can be observed at the organism level include prevention of seed germination, of root development and of the growth of the plant, and chlorosis can be seen in the leaves. At the cellular level, excess Zn can significantly alter mitotic activity, affect membrane integrity and permeability and even kill cells. Investigates showed that Zn stress on the activity of many antioxidative enzymes (APX, SOD, POD and CAT) and antioxidant contents (ascorbate and GSH) in plants [96–105].

The availability of manganese (Mn) to plants is governed by redox processes, which depend on soil's Mn reserve, pH and the availability of electrons. However, excess Mn disturbs the metabolism of plants and inhibits the plant growth. Mn causes deficiency of Fe, Mg and Ca and induces inhibition of chlorophyll biosynthesis and a decline in the photosynthetic rate. The toxic effects of heavy metals, both essential and nonessential elements, have been linked to the production of ROS. To quickly get rid of ROS, which result in the disruption of cellular metabolism due to damage by oxidative stress to important molecules, numerous enzymatic and non-enzymatic ways have evolved in living organisms. As  $Mn^{2+}$  plays a role in numerous processes, it is thought that an excess of it results in oxidative stress [106]. Cadmium (Cd) is a non-redox metal unable to participate in Fenton-type reactions. Naturally occurring amounts of Cd are normally low, however, the concentration can be significantly increased by anthropogenic activities. The impact of the uptake of Cd by living cells has been shown to be drastic, inducing oxidative stress and normally leading to cell death depending on the metal dose and time length of exposure [107]. In general, Cd in plants reduces growth, both in roots and stems, due to suppression of the elongation growth rate of the cells [94]. According to Dinakar et al. [93], cadmium is easily translocated from plant roots to above-ground tissues and potentially threatens human health. Cadmium in plants interferes with physiological processes, resulting in declined productivity. Cadmium can harness photosynthetic activity, chlorophyll content, plant growth and induce oxidative stress. ROS are efficiently eliminated by non-enzymatic (glutathione, ascorbate,  $\alpha$ -tocopherol and carotenoids) and enzymatic defence systems such as SOD, APX, POD and GR, which protect plants against oxidative damage. The detoxification of  $O_2$  occurs due to the SOD enzyme, while  $H_2O_2$  is detoxified by the enzymes CAT and PODs and thus OH radicals are not formed. In the detoxification of hydrogen peroxide from different compartments in the cell, glutathione reductase (GR) and APX are key players in the ascorbate–glutathione cycle. Glutathione is also the substrate for the biosynthesis. A constitutively high antioxidant capacity or increase in antioxidant level could prevent oxidative damage and improve tolerance to the oxidative stress established [108, 109]. Sandalio et al. [108] investigated effects of cadmium on antioxidative enzyme activity in pea. They said that the level of oxygen radicals in cells could be enhanced by a decrease of the enzymatic antioxidants involved in their detoxification, such as SOD.

It was recorded by Schützendübel and Polle [110] that antioxidative enzymes were prevented from functioning and that GSH was depleted for a short period by cadmium and other metals. It was also put forth that hydrogen peroxide accumulation resulted from the depletion in these antioxidants. These results were obtained by accessing models of antioxidative capacity. As more Cd tolerance was observed in plants when more GSH was synthesised, it can be deduced that the decrease in GSH levels is an important step for cadmium sensitivity [110]. Dong et al. [111] investigated that effect of Cd concentration in tomato seedling for antioxidative enzymes. From the results, it can be observed that POD and SOD activities significantly increased in plants that were given Cd with a concentration of 1–10  $\mu M$  and that MDA levels also showed a significant increase, indicating that oxidative stress response was the result of Cd stress in tomato plants. Tanyolac et al. [112] reported that tolerance and protective mechanisms have evolved to scavenge free radicals such as superoxide, hydroxyl radicals and peroxides generated during various metabolic reactions. Antioxidative enzymes such as APX play a key



role in controlling the cellular level of these radicals and peroxides. They found that APX activity was increased with Cu treatment [112]. Zhao et al. [113] investigated the different tolerance mechanisms to Cd stress between YSL189 and HZ903 at the seedling stage. When Cd concentration was  $>20 \mu\text{M}$  in the growing medium, the uptake rate of Cd was significantly higher in roots of YSL189 than in the roots of HZ903. When plants were supplied with 50- and  $100\text{-}\mu\text{M}$  Cd in the growing medium, there were higher Cd concentration, higher biomass and plant height, shorter roots and higher expression levels of transporter genes natural resistance associated macrophage proteins (Nramp)2, Nramp3 and zinc and iron regulated transporter (ZIP) in roots of YSL 189 compared to HZ903. The high Cd accumulation in YSL189 was partly due to the higher Cd uptake rate and higher expression levels of Nramp2, Nramp3 and ZIP in its roots. At the same time, the degree of cell injury indicated by thiobarbituric acid reactive substance showed no significant differences in roots and stems between the two genotypes. The higher activities of SOD, POD and CAT in roots and stems of YSL189 were compared to HZ903 [113].

Xiong and Wang [95] indicated that Cu phytotoxicity in *Brassica pekinensis*. Cu treatments increased electrolyte leakage and POD activity, showing a significant correlation between Cu concentration in shoots with electrolyte leakage and POD activity. Oriental melon IVF09 was used as a scion, while the pumpkin Jinxinzheng No. 3 was used as stock to research the physiological characteristics of grafted melon (*Cucumis melo*) seedlings when copper stress was induced. It was observed from the results that copper stress resulted in the inhibition of the physical characteristics of the melon seeds. In the grafted seeds, as opposed to self-rooted seedlings, an increase was seen in the levels of glucose, photosynthetic pigments, fructose, the photosynthetic parameters, biomass, the phosphate and sucrose synthase activities, acid invertase and neutral invertase. When levels of Cu decreased and the levels of P, NA and K increased nutrients were taken up more easily. The concentration of CU in the leaves decreased by 31.3%, while a 15.2% decrease was seen in roots of the grafted seedlings when the levels of copper ion ( $\text{Cu}^{2+}$ ) stress became  $800 \mu\text{M}$  and it was shown that grafting resulted in better endogenous hormone balance in the seedlings. When compared to the control, it was observed that grafted seedlings had a higher concentration of IAA and that POD activity was increased, while concentrations of ABA and maleic dialdehyde and the CAT and SOD activities became less. Thus, it could be deduced that grafting of melon seedlings was beneficial to them when under copper stress and relieved the resulting physiological characteristics from the stress, showing that the resistance of the grafted seedlings to copper stress increased due to grafting [114].

Shi and Zhu [106] indicated that the accumulation of ROS significantly increased in cucumber leaves exposed to excess Mn. It was observed that cucumber leaves in the presence of excess Mn resulted in higher activity of SOD, DHAR, POD and GR while adding SA (salicylic acid) resulted in the inhibition of the activities of APX and CAT, thus showing that different antioxidant enzymes had different changes. When the cucumber leaves were treated with SA, in the presence of excess Mn, the concentrations of the essential glutathione and ascorbate antioxidants increased [106]. Human health risk from heavy metal bioaccumulation in vegetables has been a subject of growing concern in recent years. It was observed by Kiran et

al. that when under abiotic stress, mainly heavy metal applications, the Burdur Merkez and Burdur Bucak genotypes that were salt-tolerant had a higher resistance as opposed to the sensitive genotypes. The results also showed that drought, heavy metal and salinity stress resistance was observed to have evolved in similar ways in plants [115]. Another heavy metal lead (Pb) exerts adverse effects on morphology, growth and photosynthetic processes of plants; causes inhibition of enzyme activities, water imbalance and alterations in membrane permeability; and disturbs mineral nutrition [116]. Wastewater, which is used in agriculture in order to provide growing water demand, might be included heavy metal and trace elements. Lead is one of the most hazardous heavy metals, and it causes an extensive pollution in the environment, and also it has adverse effect on the growing of plants. In the other study was conducted to evaluate the effects of Pb stress in on lettuce (*Lactuca sativa*). It was found that SOD and GR were increased with oxidative stress [115].

## 6. Conclusions

In conclusion, both the callus tissue and whole plant studies show a positive correlation between increased antioxidant activity and different abiotic tolerance. Antioxidative enzyme activities play an important role against stress. The tolerance level against salt, drought and chilling stress in callus culture can be utilised as an effective criterion in the plants with other physiological criteria. Therefore, it can be said that antioxidative defence mechanisms and effective working systems in the aspect of tolerance against stress conditions in the plants. The literature suggests that tolerant and sensitive genotypes show different responses under abiotic stress conditions, that antioxidative enzyme activities play a protective role against abiotic stress and that antioxidative defence mechanisms are effective in providing resistance to stress in plants. The results of the studies showed that the young plants of the tolerant genotypes may have better protection against stress by increasing the activity of antioxidant enzymes under different abiotic stresses.

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# Role of ABA in Arabidopsis Salt, Drought, and Desiccation Tolerance

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Additional information is available at the end of the chapter

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## Abstract

The ability of plants to respond to environmental stimuli is essential to plant survival. Absciscic acid (ABA) is a phytohormone with roles at various stages of plant development. ABA also plays a major role in mediating physiological responses to environmental stresses such as salt, osmotic, and cold stress. Plant responses to environmental stress have been widely studied in the model plant *Arabidopsis thaliana* and ABA signaling mechanisms elucidated. In general, the adaptive responses of plants to various stress conditions can be either ABA-dependent or ABA-independent. Here we focus on the role of ABA in stress signaling and abiotic stress tolerance. We describe the intrinsic mechanisms that confer stress tolerance via ABA, as well as how ABA-regulated gene products play a role in salt and drought tolerance at different stages of the life cycle. In addition, the contribution of ABA to regulation of stomatal aperture and therefore desiccation tolerance will be discussed. Understanding ABA signaling mechanisms in abiotic stress provides avenues for improving plant performance.

**Keywords:** ABA signaling, salt, drought, desiccation, Arabidopsis

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## 1. Introduction

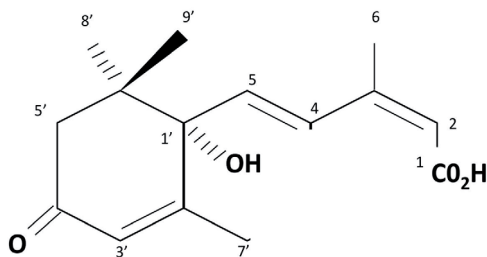
Due to their sessile nature, plants cannot avoid environmental stresses, thus they have evolved mechanisms to overcome the detrimental effects of stress. For example, plant endogenous developmental programs are modified such that structural and metabolic changes assist to overcome adverse environmental conditions such as salinity and drought. Failure to adapt to adverse environmental conditions can significantly reduce yield by impacting plant development and productivity. Abiotic stress conditions initiate a number of molecular, biochemical, and physiological changes at both the cellular and whole plant levels [1]. One major biochemical change in response to stress is elevation of abscisic acid (ABA) levels, which in turn triggers

expression of a cascade of stress-responsive genes [2]. Cellular ABA levels are induced by environmental stimuli such as light, water, and salinity stress [3].

The plant hormone ABA has been identified as a key regulator of multiple stresses. In general, the adaptive responses of plants to various stress conditions can be either ABA-dependent or ABA-independent. However, there is no clear boundary between these two pathways and there is lot of crosstalk between the pathways and the components involved. This review will focus on recent advancements in ABA-mediated stress signaling and the role of ABA in abiotic stress tolerance in the model plant *Arabidopsis thaliana*.

## 2. The phytohormone Absciscic Acid (ABA)

ABA, a sesquiterpenoid ( $C_{15}H_{20}O_4$ ) with a 15-carbon ring (Figure 1), has a variety of biological functions and is found ubiquitously across several kingdoms, including cyanobacteria, sponges, algae, lichens, mosses, and mammals [4-7]. Discovered in the 1960s and initially named dormin or abscissin, ABA is now established as a widely occurring and important plant growth regulator. Although it was initially identified as an abscission-promoting hormone, later scientists discovered that this was partly due to an indirect effect of inducing ethylene biosynthesis [8]. ABA is an important regulator of plant growth, including embryo and seed development, seedling establishment, vegetative and reproductive growth as well as promoting seed dormancy [9,10]. Seed maturation and promotion of dormancy are important in preventing preharvest sprouting. In addition, ABA has the ability to antagonize the germination promoting effects of gibberellin, regulate guard cells, and regulate stress-responsive gene expression under water-deprived conditions. ABA also has a role in plant pathogen responses in a pathosystem-dependent manner [4,5].

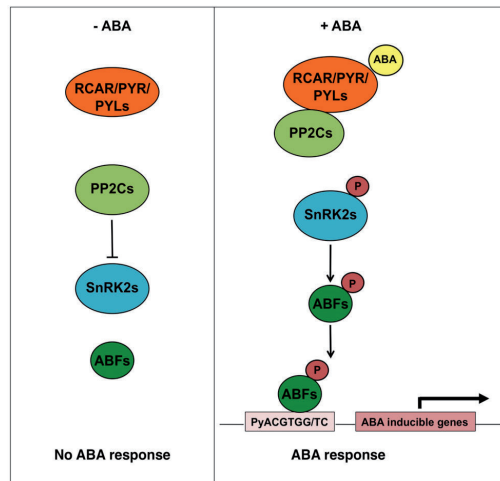


**Figure 1.** Structure of phytohormone abscisic acid S-(+)-ABA

The molecular structure of ABA has several important features that facilitate its biological functions. The side chain with the two double bonds (Figure 1) and ABA's stereocenter are two such important features. Exposure to UV light changes the conformation from active to inactive form [5].

## 2.1. ABA signaling in plants

Although ABA has a broad range of functions in plant growth and development, its main function is to regulate plant water balance and osmotic stress tolerance [11]. Thus, understanding ABA signaling is essential to improving plant performance. Genetic screens in *Arabidopsis thaliana* identified many downstream ABA signaling components. Recent findings in the field of ABA signaling reveal a unique hormone perception mechanism (Figure 2) where ABA binds to the ABA receptors Regulatory Components of ABA Receptor/Pyrabactin Resistance Protein1/PYR-like Proteins (RCAR/PYR1/PYLs). RCAR/PYR/PYL proteins belong to the START-domain superfamily and have soluble ligand-binding properties. RCAR/PYR/PYL receptors are found in the cytoplasm as well as in the nucleus. ABA binding to RCAR/PYR/PYLs leads to inactivation of type 2C protein phosphatases (PP2Cs) such as ABSCISIC ACID INSENSITIVE 1 (ABI1) and its close homolog ABI2 [12]. All 14 members of the RCAR family of proteins bind to ABA and interact with PP2Cs. Except for RCAR7/PYL13, all the other RCAR members are positive regulators of ABA signaling. Among the 80 PP2Cs identified in Arabidopsis, six out of nine clade A PP2Cs act as negative regulators of ABA signaling [13]. These phosphatases and RCAR/PYR1/PYLs function as co-receptors and form a high-affinity ABA-binding site. Inactivation of PP2Cs causes suppression of PP2C-mediated dephosphorylation of Sucrose nonfermenting Kinase-1-Related protein kinase 2s (SnRK2s), which are important positive regulators of ABA signaling. As a result, activated SnRK2s target ABA-dependent gene expression and ion channels [5,11]. Table 1 summarizes the major positive and negative regulatory elements in the ABA signaling pathway. Phosphorylated SnRK2s subsequently phosphorylate ABA-responsive element Binding Factors (ABFs), which are basic leucine zipper transcription factors that bind to ABA-Responsive Elements (ABRE) (PyACGTGG/TC), the major *cis*-element in the promoter region of downstream genes that are induced by ABA [19,20].



**Figure 2.** Main components in the core ABA signal transduction pathway

Signaling component	Regulation	Expressed	References
Group A PP2Cs	Negative regulators		
ABA INSENSITIVE 1/2 (ABI1/2)		Various tissues and developmental stages	[13-16]
ABA HYPERSENSITIVE GERMINATION 1 (AHG1)			
HYPERSENSITIVE TO ABA 1/2 (HAB1/2)			[17]
SnRK2 subgroup III	Positive regulators		
SRK2D/SnRK2.2		Seeds and vegetative tissues	[18]
SRK2I/SnRK2.3		Seeds and vegetative tissues	[18]
SRK2E/OST1/SnRK2.6		Expressed in guard cells and involved in stomatal closure	[15]

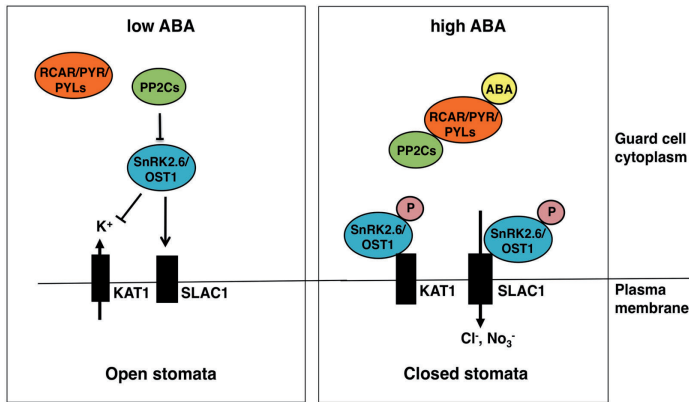
**Table 1.** Major positive and negative regulators of ABA signaling

Therefore, the ABA signaling complex/ABA signalosome is comprised of three major components: (a) RCAR/PYR/PYLs; (b) PP2Cs; and (c) SnRK2s assembled as a double negative regulatory system [7]. In the absence of ABA, PP2Cs dephosphorylate SnRK2s inhibiting kinase activity and thereby preventing downstream gene expression (Figure 2). Several studies showed that these core components are essential for ABA signaling. For instance, Fujita *et al.* [21] showed ABA signaling is completely blocked and *ABF* genes showed reduced expression in the *snrk2.2/2.3/2.6* triple null mutant but not in single or double mutants. In addition, reduced phosphorylation of other bZip transcription factors such as ABSCISIC ACID INSENSITIVE 5 (ABI5), which is a dormancy promoting transcription factor, was also observed [18,22].

In guard cells (Figure 3), ABA binds to the PYR/PYL/RCAR receptor-PP2C complex and blocks its phosphatase activity. Consequently, activated protein kinase SnRK2.6/OPEN STOMATA 1 (OST1) phosphorylates and regulates the key target ion channels, SLOW ANION CHANNEL ASSOCIATED 1 (SLAC1) and K<sup>+</sup> CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1). SnRK2.6/OST1 acts as a positive regulator of stomatal closure where it activates anion channel SLAC1 and inhibits cation channel KAT1 [23-25].

**2.2. ABA-binding proteins and alternate ABA receptors**

Identification of putative ABA receptors using forward genetic approaches was not successful for a long time due to genetic redundancy of the genes encoding ABA receptor proteins. However, biochemical approaches leading to purification and analysis of high-affinity ABA-binding proteins have been successful in identification of potential ABA receptor classes [5,26]. Some of these potential ABA receptors are cytosolic while others are on the cell surface,



**Figure 3.** ABA signaling in guard cells

indicating there are extracellular as well as intracellular sites of ABA perception. Studies indicate that there can be multiple ABA receptors at different locations of the cell [27].

#### 2.2.1. ChlH/ABAR (*H* subunit of the chloroplast magnesium chelatase/ ABA receptor)

The primary function of ChlH is chlorophyll synthesis. ChlH was initially identified as an ABA-binding protein in broad bean (*Vicia faba*) and the Arabidopsis protein was named as ABAR. Later it was found that binding of ABA to ChlH/ABAR depends on the stereochemistry and it specifically binds to only (+) ABA to mediate ABA responses. Although ChlH/ABAR is localized in the chloroplast envelope, it functions as a negative regulator of ABA signaling in the nucleus. The cytosolic C-terminus of the ABAR interacts with WRKY transcription factors (WRKY 18, 40, 60) which act as transcriptional repressors, repressing ABA-responsive gene expression in the nucleus. Binding of ABA with ChlH/ABAR promotes interaction with WRKYs, preventing them from repressing downstream genes such as *ABI5* and *DREB2* [28-31]. Thus, it has been proposed that ChlH mediates nuclear-chloroplast signaling. However, another research group has not been able to reproduce these results using *wrky* loss of function mutants. In addition, barley ChlH does not bind to ABA and ChlH loss of function mutants do not show any impaired ABA responses. Despite its ABA-binding properties in Arabidopsis, it is not confirmed whether ChlH functions as an ABA receptor (reviewed in [5,27]). However, ChlH/ABAR mediates ABA-induced stomatal closure and ABA inhibition of blue-light-mediated stomatal opening. In addition, ChlH/ABAR has a role in ABA-mediated fruit ripening in peach and strawberry (reviewed in [31]).

#### 2.2.2. GTG1/GTG2 (*G* protein coupled receptor type *G* protein 1 and 2)

G protein coupled ABA receptors are plasma membrane localized cell surface receptors that are widely expressed in plants. Both GTG1 and GTG2 showed specific and saturable ABA-

binding activity in direct ABA-binding assays. GTGs have GTPase activity and GDP bound GTGs have enhanced ABA-binding ability, which in turn initiates ABA signaling. GTG1/2 bind with GPA1 (G-PROTEIN  $\alpha$  SUBUNIT 1), which abolishes its GTPase activity and represses ABA binding. GTP bound GPA1 represses ABA signalling. However, the downstream components of this pathway are not characterized yet [31,32].

### 2.3. Recent studies on ABA perception and signaling mechanisms

Recent findings that several ABA receptors exist in different parts of the cell provide evidence that ABA is active in a variety of subcellular compartments. ABA synthesis enzymes are present in different compartments, suggesting that ABA synthesis occurs in different parts of the cell and that these ABA levels contribute to overall ABA homeostasis. For example, the ABA biosynthesis enzyme AtABA1 is localized in the chloroplast, whereas AtABA2 is in the cytosol [33,34]. It has also been proposed that ABA produced in cytoplasm, plastids, vacuole, and other subcellular organelles may have different physiological roles initiated by signaling networks via different ABA receptors in each specific compartment [35].

Takeuchi *et al.* [36] identified a potential ABA analog AS6 that can inhibit the activity of PYLs. X-ray crystallography studies showed the structure of ABA facilitates the binding of ABA to PYR/PYL/RCAR receptors and thereby inhibits interaction with PP2Cs. The AS6 ABA analog was able to block PYL-PP2C interaction, indicating that binding of ABA to PYL receptors initiates ABA responses by repressing PP2Cs.

Inhibition of PP2Cs results in autoactivation of SnRK2 kinases and thereby positive regulation of ABA signaling. Recently, the crystal structures of SnRK2.3 and SnRK2.6 were elucidated, providing evidence that kinase activation is a two-step mechanism as well as details of how the ABA signal is transmitted to downstream components [37]. This study also showed that autophosphorylation of SnRK2.6 is more efficient than that of SnRK2.3.

Lumba *et al.* [20] did a comprehensive transcriptomic data analysis in order to generate a mesoscale ABA signaling network. They showed that there are 3 main kinase hubs, MAP3K04, SnRK3.15, and SnRK3.22, that interact with PP2Cs and these kinases act as negative regulators of ABA response, in contrast to the SnRK2s involved in ABA signaling. SnRK3.15 and SnRK3.22 also interact with a large number of transcription factors and may have a role in overall ABA responses in the plant [20].

## 3. ABA in stress signaling

In plants and other organisms, such as algae, cyanobacteria, and fungi, ABA levels tend to increase with exposure to stress, suggesting a potential role of ABA in stress signal transduction [20]. Exogenous ABA application mimics stress conditions in plants and provides a useful means to study the effect of ABA on stress signaling and tolerance [38]. ABA distributes throughout the plant as an inactive glucose sugar conjugate and is converted to the active form by  $\beta$ -glucosidase [4]. ABA acts as an endogenous messenger and salt and drought stress signal



transmission to initiate downstream gene expression occurs mainly via ABA signaling. However, cold stress signal transduction occurs in an ABA-independent manner via the C-REPEAT/DEHYDRATION RESPONSIVE ELEMENT BINDING FACTORS (CBFs/DREBs) signaling pathway [39].

A large number of ABA-responsive genes have a common *cis*-element called the ABRE element in their promoter regions. An ABRE together with a Coupling Element (CE) results in ABA induction of gene expression [19]. The ABA RESPONSIVE ELEMENT BINDING FACTOR (AREB/ABF) family of transcription factors are the major transcription factors that regulate ABA-induced gene expression. AREB/ABFs are bZIP transcription factors and their expression is induced by ABA and other potential stress conditions [40]. Different ABFs are induced by ABA at different rates. For instance, *ABF2*, *ABF3*, and *ABF4* are induced faster than *ABF1*. Moreover, *ABF1* is induced only by cold stress, whereas *ABF2* and *ABF3* are induced by salt stress. *ABF4* levels are induced by salt, drought, and cold stress, suggesting that distinct ABFs have roles in various ABA-dependent stress-responsive pathways [40].

There are nine Group A bZIP transcription factors implicated in ABA signaling and they are subdivided into two groups based on where they are mainly expressed. The *ABI5/AtDPBF* family of genes includes *ABSCISIC ACID INSENSITIVE 5 (ABI5)*, *ENHANCED EM LEVEL (EEL)*, and *AREB3* and are expressed in the seed during seed maturation [41]. Other AREB/ABF transcription factors are mainly expressed in vegetative tissues [40].

SnRK2 III is the major subfamily of SnRKs involved in abiotic stress responses. SnRK2 protein kinases phosphorylate AREB/ABFs and regulate their function in ABA-regulated gene expression under stress conditions [10]. SnRK2.6/OST1 is an important regulator of stomatal closure in drought stress. The role of SnRK2.2 and 2.3 is mainly to transmit the ABA signal to inhibit seed germination and seedling growth in response to stress. A decuple *snrk2* mutant in Arabidopsis, which carries mutations for all 10 SnRK2 members, was hypersensitive to osmotic stress and also defective in ABA accumulation and ABA-induced gene expression under osmotic stress, indicating the critical role of SnRK2 kinases in osmotic stress signaling and tolerance. Moreover, the *snrk2.2/3/6* triple mutant had impaired accumulation of proline, which is a compatible osmolyte [42].

### 3.1. ABA and abiotic stress tolerance

In *Arabidopsis thaliana*, about 10% of the genome consists of ABA-regulated genes. Approximately half of these genes are ABA-induced genes and the rest are ABA-repressed. ABA-induced genes code for proteins that confer stress tolerance such as dehydrins, detoxifying enzymes of reactive oxygen species, regulatory proteins (transcription factors, protein kinases, phosphatases) and enzymes required for phospholipid signaling. Genes that are repressed by ABA are mostly related to growth [5]. ABA biosynthesis mutants identified in Arabidopsis [43] and other crop plants [44] wilt and die under prolonged salt and drought stress, suggesting ABA plays an important role in osmotic stress tolerance.

Drought and high salinity generate osmotic stress in plant cells. Endogenous ABA levels are elevated in response to osmotic stress, which in turn coordinates the plant's response to

reduced water availability. In addition, seed maturation and post-germinative growth creates cellular dehydration stress, which again results in accumulation of ABA in cells [45]. The role of ABA in drought and salt stress is twofold: water balance and cellular dehydration tolerance. Water balance is achieved through guard cell regulation and the latter role by induction of genes that encode dehydration tolerance proteins in nearly all cells. ABA accumulation is induced by osmotic stress and this is as a result of activation of ABA biosynthesis as well as inhibition of ABA degradation [46]. Thus, ABA-mediated adaptive stress responses of plants to environmental stimuli occur via ABA-responsive gene expression and regulation of stomatal pore size. ABA-responsive gene expression involves various transcription factors, ABA receptors, secondary messengers, protein kinase/phosphatase cascades, and chromatin remodeling factors [45].

Both drought stress and salinity stress upregulate osmotic stress responsive genes that are ABA-inducible. Most of the high-salinity-induced genes are also induced by drought, suggesting there is overlap between salt and drought stress tolerance mechanisms [6]. A large number of transcription factors are induced by multiple stress conditions. AREB1/ABF2, AREB2/ABF4, ABF3, and MYB41 are some of the main transcription factors that are induced by both salt and drought in vegetative tissues [45].

Drought and salt stress results in osmotic imbalance; thus, salt and drought stress tolerance mechanisms aim at restoring cellular homeostasis. These mechanisms are adaptive responses that create either stress tolerance or avoidance of stress conditions. Modifications in metabolic pathways, synthesis of new proteins, changes in ion uptake, and free radical scavenging are some of the stress responses at the cellular level, immediately followed by stress signal transduction [47]. High ABA levels in cells result in synthesis of storage proteins, desiccation tolerance, and dormancy via inhibition of seed germination [22]. In the plant as a whole, key adaptive responses include induction of stomatal closure as well as control of seedling growth and lateral root formation. While the balance between ABA and auxin levels slightly affects primary root growth, ABA represses lateral root formation while auxin promotes it [4,48].

Inhibition of seed germination under abiotic stress is another function of ABA. Seed germination occurs when there is a balance between germination-promoting gibberellin and dormancy-promoting ABA. During late stages of maturation, seeds accumulate ABI5 which in turn activates transcription of LATE EMBRYOGENESIS ABUNDANT (LEA) proteins. LEA proteins confer osmotolerance to the embryo. ABA is necessary for activation of ABI5 via SnRK2.2 and SnRK2.3 phosphorylation of ABI5 [49,50]. When seeds are in unfavorable environmental conditions, elevated endogenous ABA levels results in ABI5 accumulation, preventing seeds from germinating.

### 3.1.1. *ABA and salt tolerance*

Salt stress severely impacts plant growth by affecting metabolic processes and photosynthetic efficiency. NaCl initially induces osmotic stress and eventually accumulation of both Na<sup>+</sup> and Cl<sup>-</sup> ions generates ionic stress [51]. However, some responses are salt-specific and distinct from responses to osmotic stress (reviewed in [52]). High salinity in the soil is first sensed by the plant roots. Salt and drought stress induce a rapid increase in cytosolic Ca<sup>2+</sup> levels in the root

cells.  $\text{Ca}^{2+}$  acts a second messenger, inducing salt- and drought-responsive genes [53,54]. Hyperosmotic stress is coupled with  $\text{Ca}^{2+}$  signaling and Reactive Oxygen Species (ROS) signaling, thereby inducing a cascade of signaling events, which results in downstream gene expression [52].

Biochemical and molecular mechanisms of salt tolerance in plants include exclusion of salt ions, production of suitable osmolytes, changing the structure of the membranes to control ion uptake, and induction of enzymes that produce antioxidants and phytohormones. To manage salt or drought stress, cellular ABA levels increase dramatically. The plant cuticle has been shown to mediate stress signaling as well as ABA biosynthesis and signaling. In addition to its primary function, providing mechanical support to the cell wall and plasma membrane, the cuticle has been implicated in osmotic stress regulation. CED1 (9-CIS EPOXYCAROTENOID DIOXYGENASE DEFECTIVE 1) is an essential protein in cuticle biogenesis. *ced1* mutants are sensitive to osmotic stress, as they are unable to induce ABA biosynthesis in response to osmotic stress [55].

ABA regulates root growth and architecture in plants under stress. Duan *et al.* [56] showed that salt has a strong inhibitory effect on lateral root growth, while primary roots are less sensitive to salt stress. They also showed that endogenous ABA signaling affects root system architecture under stress conditions using ABA biosynthesis mutants (*aba1*, *aba2*) as well as signal transduction mutants such as *abi1*. Salt stress results in elevated levels of ABA exclusively in lateral root cells and induces a quiescent period in postemergence lateral roots. Lateral roots in a quiescent stage form a thick, well-developed Casparian strip, which acts as a barrier to reduce diffusion of  $\text{Na}^+$  ions through the endodermis. In the presence of  $\text{Na}^+$  ions, endodermal cells activate ABA signaling and arrest growth so that lateral roots do not elongate into high saline environments. Therefore, ABA is an important signaling molecule in suppressing lateral root growth during salt stress [56].

ABA regulates expression of many salt-stress-responsive genes via transcription factors that are elevated in response to salt. For instance, ABF2/AREB1, ABF3, ABF4/AREB2, ABRE BINDING PROTEIN 9 (ABP9), and MYC/MYB, WRKY, and APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) are some of the salt-stress-responsive transcription factors that enhance stress tolerance. A recent study showed that the PYL8/RCAR3 ABA receptor has a role in ABA-mediated inhibition of primary root growth and also recovery of lateral root growth on exposure to ABA. PYL8/RCAR3 combines the action of ABA and auxin through direct interaction with MYB transcription factors during growth recovery of postemergence lateral roots [48,57].

There are proteins in the cell that are produced in an ABA-dependent manner that have a role in osmotic tolerance. For example, ABI5 activates transcription of LEA proteins. LEA proteins are highly hydrophilic small proteins shown to have an osmoprotectant role against cellular dehydration during late embryogenesis. LEA proteins also have a role in salt stress tolerance [58]. Due to their hydrophilic nature, LEA proteins can sequester ions accumulating in the cell, as well as act as chaperones and retain water molecules to prevent protein aggregation and inactivation of cellular enzymes [59]. In Arabidopsis, 51 LEA proteins have been identified that belong to nine different groups [60]. Jia *et al.* [61] showed overexpression of AtLEA14,

which belongs to the LEA group 2 proteins, overactivates salt-stress-inducible genes such as *RD29B*, which encode dehydration protective proteins, and subsequently confers salt tolerance in *Arabidopsis*.

In addition, ABA has been implicated in histone H3 acetylation and methylation, thereby regulating stress-inducible gene expression at the epigenetic level. Chen *et al.* [62] showed that histone modifications by HISTONE DEACETYLASE 6 (HDA6) are involved in inhibition of seed germination, salt stress responses, and ABA- and salt-mediated gene expression in *Arabidopsis*.

### 3.1.2. ABA and drought tolerance

Drought is lack of water in the soil. Drought stress in plants arises due to water deficit conditions and results in removal of water from the cell membranes, disrupting the lipid bilayer structure. In addition, protein denaturation and accumulation of cellular electrolytes results in disruption of cellular metabolism [63]. Therefore, drought causes osmotic stress, and osmotic stress causes dehydration and inhibition of water uptake in plants. ABA accumulates under osmotic stress conditions and plays an important role in the stress response and tolerance of plants. In addition to autoactivation of SnRK2s by inhibition of PP2Cs in the ABA signaling cascade, hyperosmotic stress activates SnRK2s [64]. SnRK2 kinases are a major component of the osmotic stress signaling pathway. The *Arabidopsis* triple mutant *snrk2.2, snrk2.3, snrk2.6* shows severe drought intolerance and ABA-insensitivity [42]. Also ABF2, ABF3, and ABF4 act as transcriptional activators in mediating ABRE-dependent ABA signaling, which confers drought tolerance in vegetative tissues [40].

ABA induces expression of many transcription factors as well as genes that encode enzymes in the synthesis of osmoprotectants [65]. Osmolytes are compatible solutes such as amino acids (proline), sugar alcohols (mannitol, pinitol), and other sugars that accumulate without disrupting the function of proteins. Osmolytes make an osmotic adjustment facilitating a favorable water potential gradient and promote stress tolerance [66].

Dehydrins and LEA-like proteins act as cellular chaperones that protect cellular membranes and macromolecules in the cell [2]. During seed maturation seeds undergo dehydration stress. LEA proteins accumulate in the embryo as a result of osmotic stress and their functions include protection of enzymes, lipids, and mRNAs from dehydration. LEA proteins have been found to protect mitochondrial membranes from damage. LEA proteins are produced in an ABA-dependent and ABA-independent manner under osmotic stress [47,58].

Under moderate water stress conditions plant root growth has to be maintained in order to keep the plants alive. ABA accumulates under moderate water stress and mediates auxin transport in the root tip, which enhances the proton pumps in the plasma membrane. Proton secretions in the root tip play an important role in primary root growth and root hair development under moderate drought stress [67].

Based on the critical water level, drought tolerance is considered to be mechanisms that confer tolerance to moderate dehydration. Further dehydration requires desiccation tolerance mechanisms in order to restore the ability of cells to rehydrate successfully [68].

### 3.1.3. ABA and desiccation tolerance

Water loss results in a change in turgor pressure that affects the cell walls. Desiccation tolerance is defined as evolution of cell walls that can withstand extensive water loss without damaging its structure or polymer organization. Desiccation tolerance mechanisms aim to restructure the cells walls and maintain normal growth under water stress conditions [69].

Regulation of the stomatal pore is crucial in adapting plants to abiotic stress by reducing extensive water loss. Stomatal opening and closing occurs as a result of turgor pressure differences in the surrounding guard cells [47]. In response to water stress, ABA concentration is increased in the guard cell cytoplasm and apoplast, which results in a decrease in the turgor pressure due to activation of the K<sup>+</sup> outward rectifying channel and inhibition of the K<sup>+</sup> inward rectifying channel (KAT1 and 2). ABA also induces the anion channel SLAC1 resulting in release of anionic organic acids from the vacuole to the cytoplasm [70]. Reduced turgor pressure initiates closure of stomata as a mechanism of minimizing water loss from the plant. ABA levels rise in leaves immediately following water stress. CHLH/ABAR has been proposed as the chloroplast ABA receptor that links ABA signaling within the chloroplast with ABA signaling in the nucleus. Overexpression of CHLH promotes stomatal closure and thereby desiccation tolerance [71].

SnRK2 OPEN STOMATA 1 (OST1) is a key SnRK2 protein kinase involved in regulation of the stomatal aperture by movement of guard cells during ABA signaling [72]. OST1 is activated by ABA, low humidity, and osmotic stress and is an important kinase found in guard cells preventing rapid water loss. Loss of function mutants of SnRK2 do not exhibit ABA-mediated stomatal closure activity and showed a wilted phenotype under dehydration stress conditions [72,73]. Also SnRK2.6/OST1 physically interacts with ABI1 and ABI2. ABI1 is required for ABA-dependent activation of OST1 and both ABI1 and ABI2 are required for osmotic-stress-induced activation of OST1 [15]. Thus, SnRK2.6/OST1 acts as a positive regulator in ABA-induced stomatal closure. Moreover, Yoshida *et al.* [73] showed that OST1 also positively regulates stress-responsive genes such as *RD29B* and *RD22*.

Reactive Oxygen Species (ROS) have also been identified as secondary messengers in ABA signaling in guard cells. In Arabidopsis, two partially redundant guard cell expressed NADPH oxidase catalytic subunit genes, *AtRbohD* and *AtRbohF*, were found to be involved in ABA signaling in guard cells, ABA-induced stomatal closure and ROS production, ABA activation of Ca<sup>2+</sup> permeable channels in the plasma membrane of guard cells, and increasing cytosolic Ca<sup>2+</sup> levels in response to ABA. Thus, these two genes act as positive regulators of ABA signal transduction [74]. Sirichandra *et al.* [72] provided biochemical evidence that OST1 protein kinase physically interacts with AtRbohF NADPH oxidase and phosphorylates it.

## 4. Conclusions

ABA has a wide range of functions from plant development to biotic and abiotic stress signaling and tolerance. The primary functions of ABA in salt, drought, and desiccation

tolerance act via inhibiting seed germination, altering root architecture, and inducing stress-responsive genes as well as gene products that act as osmoprotectants. ABA signaling cascades and stress tolerance mechanisms studied in *Arabidopsis* provide insight into application of stress tolerance strategies to commercial crops. While ABA is not the only plant hormone involved in stress responses, many of these responses occur in an ABA-dependent manner, indicating the importance of ABA in plant stress response and tolerance.

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## Breeding for Stress Tolerance

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# Rootstock Breeding for Abiotic Stress Tolerance in Citrus

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Additional information is available at the end of the chapter

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## Abstract

*Citrus* species are the most widely produced fruit crops in the world. Citrus fruits are mainly produced in coastal areas in several countries as well as Mediterranean region, and production in these regions is affected by both biotic and abiotic stresses, including drought, extreme temperature, salinity, citrus canker, citrus tristeza virus, citrus greening, and others. The use of rootstocks in fruit production includes not only stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metals, nutrient stress, water stress, and alkalinity. There is extensive genetic diversity in citrus which provides several materials to be used as rootstocks against abiotic stress. In this work, we tried to provide an overview of the abiotic stresses in citrus by combining literature with our studies, role of citrus rootstocks commercially used against abiotic stresses and rootstock breeding in citrus.

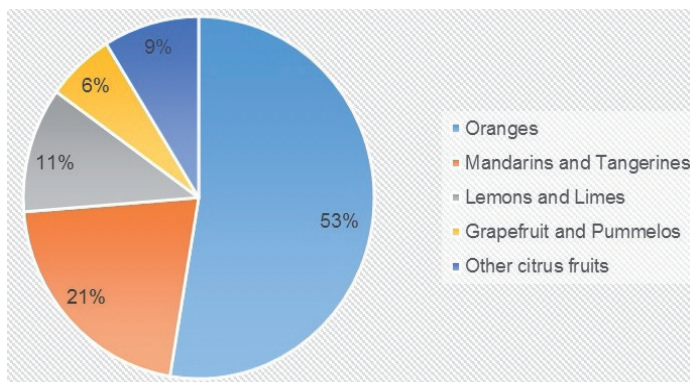
**Keywords:** Citrus, rootstock, salinity, alkalinity, drought, breeding

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## 1. Introduction

Citrus is the most important tree fruit crop in the world, and citrus fruits are regarded as major household items in more than 100 countries around the world as well as the world juice industry which is also led by citrus juices. Citrus industry is regarded as a leading industry in some regions, such as the mountainous regions of China and coastal plains in several countries, such as California and Florida in USA, Valencia in Spain, and Adana in Turkey. According to the data published in 2013, the world's total citrus fruit production is 135.761.181 tons [1], which consists of 71.445.352 tons of oranges (*Citrus sinensis* (L.) Osb.), 28.678.213 tons of mandarins (*Citrus reticulata* Blanco), 15.191.482 tons of lemons (*Citrus limon* Burm. F.) and limes (*Citrus latifolia* Tan. and *Citrus aurantifolia* Swingle), 8.453.446 tons of grapefruits (*Citrus paradisi* Macf.) and pummelos [*Citrus maxima* (Burm.) Merr.], and 11.992.686 tons of other citrus fruits (Figure 1).

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**Figure 1.** World's citrus production quantity (tons)

The role of citrus fruits in providing nutrients and medicinal value has been recognized since ancient times. Citrus fruits, belonging to the genus *Citrus* of the family Rutaceae, are well known for their refreshing fragrance, thirst-quenching ability, and providing adequate vitamin C as per recommended dietary allowance. In addition to ascorbic acid, these fruits contain several phytochemicals, which play the role of nutraceuticals, such as carotenoids (lycopene and  $\beta$ -carotene), limonoids, flavanones (naringins and rutinoid), and vitamin B complex and related nutrients (thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, folic acid, biotin, choline, and inositol). These substances greatly contribute to the supply of anticancer agents and other nutraceutical compounds with antioxidant, inflammatory, cholesterol, and allergic activities, all of them essential to prevent cardiovascular and degenerative diseases, thrombosis, cancer, atherosclerosis, and obesity. In spite of these beneficial traits, there is still a major need to improve fruit quality to meet current consumers' demands [2, 3].

Being vegetatively propagated, a citrus tree is normally composed of the rootstock and scion. Rootstocks play an important role in the rapid development of citrus in the world as well as breeding new cultivars. The necessity of using rootstocks for citrus fruits is to have a profitable production against some limiting factors such as climate, bad soil conditions, and diseases. Besides these factors, the use of citrus rootstocks provides a large number of choice to the growers to increase fruit quality and yield, obtain early fruiting and uniform cropping, avoid juvenility, controlling the tree size, have the opportunity for high-density planting, etc. These factors give many economic important advantages to the growers and, as a result, the citrus fruits are the most produced fresh fruits in the world for several decades [4]. Choosing a rootstock is an important decision, and local climatic and soil conditions are important factors in rootstock selection. Although any citrus variety can be used as a rootstock, some of them are better suited to specific conditions than the others [5, 6]. Some characteristics in a desirable citrus rootstock should be listed such as a good adaptation to all kinds of soils, tolerance to salinity, iron chlorosis, flooding, drought, high affinity with commercial species/cultivars, high yields of good fruit quality, reduced tree size, resistance to citrus tristeza virus (CTV), resist-



ance to citrus blight, resistance to fungal diseases affecting citrus (*Phytophthora* spp., *Armillaria mellea*, etc.), and resistance to nematodes.

Citrus production is affected by both biotic and abiotic stresses, including drought, extreme temperature, salinity, citrus canker, citrus tristeza virus, citrus greening, and others. These stresses can severely influence growth and development of both rootstocks and/or scions of citrus trees, thus reducing both fruit production and fruit quality. The use of rootstocks in fruit production includes not only stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metals, nutrient stress, water stress, and alkalinity [7]. Soil salinity is a major factor reducing crop production among the world as well as citrus production. Responses to salinity in terms of citrus production are affected by the amount of irrigation, climate, soil type, and fertilization [8]. Citrus rootstocks differ in terms of their tolerance to salinity conditions; many soils and sources of water contain high amounts of salts that can inhibit the growth and yield salt-sensitive citrus [9]. The high level of bicarbonate ions in the soil affects metabolic processes in roots and leaves, decreasing soil and plant Fe availability, leading to the condition known as lime-induced iron chlorosis. The most evident effect of Fe chlorosis is a decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls, and production of yellow, chlorotic leaves resulting as a decrease in fruit yield and quality [10, 11, 12]. Drought is considered as the principal factor that limits global agricultural production, among environmental constraints. Species greatly differ in the ability to overcome water deficiency. Drought in citrus trees causes reductions in stomatal conductance ( $g_s$ ), leaf transpiration rate ( $E$ ), and net  $CO_2$  assimilation; decreases fruit quality and yield in long-term periods of stress; and increases fruit abscission [13].

Although the sour orange has many excellent horticultural advantages in terms of abiotic stress, it has a very important disadvantage for its susceptibility to citrus tristeza virus. This problem has severely reduced the use of this rootstock in many places especially the Western Mediterranean. Castle and Gmitter [14] reported that sour orange no longer has a secure place in today's rootstock portfolios because of its susceptibility to CTV. Castle [15] indicated that sour orange is an excellent rootstock for areas free of CTV. Ollitrault et al. [16] reported that the arrival of tristeza radically called into question of using sour orange in the Mediterranean area, whereas it had been almost the only rootstock in the region.

The need to produce stress-tolerant crops was evident even in ancient times [17]. Searching rootstock alternative to sour orange keeps on all over the world. In addition, it is a known fact that every rootstock cannot be used in every ecology. Due to the limiting effects of different ecological factors and diseases, a rootstock which is suitable for a country could not succeed in another one. Accordingly, the rootstocks that will be offered as alternative to sour orange is supposed to show some characteristics such as tolerance to lime-induced iron chlorosis in calcareous soils, tolerance or resistance to *Phytophthora citrophthora*. In addition to these, the alternative rootstocks should have CTV tolerance which does not exist in sour orange. On the contrary, all the required traits are present in the citrus germplasm. For instance, *Poncirus trifoliata* has tolerance to mainly biotic stress and cold hardness and some *Citrus* species have

adaptation to both abiotic and biotic stresses. However, the complexity of citrus biology and genetics makes it difficult to combine them through traditional breeding.

Consequently, in this chapter, we try to provide an overview of the abiotic stresses in citrus, role of citrus rootstocks commercially used against abiotic stresses, and rootstock breeding for tolerance to abiotic stress.

## 2. Types of abiotic stresses in citriculture

Many different citrus genotypes are commercially grown in a wide diversity of soil and climatic conditions; therefore, trees are subjected to important abiotic and biotic stresses that limit the production and, in some instances, the use of certain rootstocks and varieties [18]. Citrus trees are subjected to several abiotic constraints such as acid, alkaline, and salty soils; flooding and drought; and freezing and high temperatures.

Related to the global warming, drought problems have occurred in many countries. In addition, salinity became a major cause for citrus production in the coastal regions of Mediterranean by increasing the use of fertilizers and decreasing precipitations. Salinity and drought in the calcareous soil of Mediterranean region can lead to major problems in citriculture in terms of fruit yield and quality. Thus it is necessary to breed new rootstocks that are genetically tolerant to abiotic stress conditions and alternatives to existing rootstocks.

In a recent work, we have tried to handle three common abiotic stresses (salinity, drought, and alkalinity) occurring in citriculture especially in the coastal Mediterranean region by combining the literature with the studies conducted at Çukurova University.

### 2.1. Salinity

Salinity is a major environmental factor affecting the performance of many crop plants and reducing agricultural productivity [19, 20]. It is estimated that more than a third of all of the irrigated soils in the world is affected by salinity. The loss of farmable soils due to salinization is directly in conflict with the needs of the world population which is increasing continuously. Salt stress is a major stress problem in arid and semiarid regions and irrigated areas. Almost 7% of the world land area, 20% of the cultivated land, and nearly half of the irrigated land are affected by high salt concentrations [21].

Salinity affects the crop during both the vegetative and the reproductive stage and therefore causes reduction in plant growth and development with low water potential in the root medium (osmotic effect), too high internal ion concentration (ion excess/toxicity), and nutritional imbalance by depression in uptake and/or shoot transport (ion deficiency). Most of the salt stress in nature is due to sodium salts, particularly NaCl [22, 23]. High concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in the root medium saturation depress nutrient-ion activities and produce extreme ratios of  $\text{Na}^+/\text{Ca}^{2+}$ ,  $\text{Na}^+/\text{K}^+$ ,  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , and  $\text{Cl}^-/\text{NO}_3^-$  [24]. As a result, plants become susceptible to osmotic and specific ion injury as well as nutritional disorders that may result in reduced yield and quality. These processes may be occurring at the same time, but whether they

ultimately affect crop yield and quality depends on the salinity level, composition of salt, exposed period to salinity, the crop species and cultivars, the growth stage of plants, and a number of environmental factors [25, 26, 27, 28]. When the salt concentration reaches a harmful level for plant growth, a salinity condition is said to have developed. The degree to which growth and normal metabolism can be maintained is described as salt tolerance. Salt tolerance of vegetable crops varies considerably among species and depends upon the cultural conditions under which the crops are grown. Soil, water, plant, and environment can affect the salt tolerance of a plant. Therefore, plant response to a given salt concentration cannot be predicted on an absolute basis but on relative performance [29].

The effects of salinity on plants are evidenced by a severe reduction in plant growth and yield and, if the saline conditions persist, plant death can occur [30]. Salinity causes a deficiency of water in plant tissue, and low water potential reduces growth by inhibiting cell division and cell expansion [31]. The reduction in growth is mainly due to an osmotic effect of the accumulation of salts near the root zone, whereas the buildup of toxic saline ions in plant tissues is responsible for the progressive impairment of several physiological processes [20].

Osmotic effects resulting from salinity may cause disturbances in the water balance of the plant, including a reduction of turgor and an inhibition of growth, as well as stomatal closure and reduction of photosynthesis [32, 33]. The primary effect of high salt concentration in plants is stomatal closure. This causes a low transpiration rate and reduces the CO<sub>2</sub> availability for photosynthesis [34]. Hussain et al. [35] indicated that salinity reduced the photosynthetic availability of some citrus species and genera. As a result, plants become susceptible to osmotic and specific ion injury as well as to nutritional disorders that may result in reduced yield and quality.

Many researchers so far have reported citrus trees as salt-sensitive plants [30, 36, 37]. Salinity reduces citrus tree growth and fruit yield [9]. Growth reduction and some physiological and biochemical disturbances due to excessive concentrations of Cl<sup>-</sup> and Na<sup>+</sup> in leaves are the main problems that are caused by salinity stress [37]. Also, salt stress has a dramatic impact on the citrus industry by decreasing the growth of trees and fruit yield and quality. Salinity may also cause nutrient deficiencies or imbalances, due to the competition of Na<sup>+</sup> and Cl<sup>-</sup> with nutrients such as K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and NO<sub>3</sub><sup>-</sup>. In addition to osmotic effect, high K<sup>+</sup> concentration in a salinized nutrient solution increased the absorption of Cl<sup>-</sup> citrus roots. Salt tolerance in citrus has been linked to the exclusion of toxic ions from the shoot [38]. Thus, citrus rootstocks have a great influence on the amount of Cl<sup>-</sup> and/or Na<sup>+</sup> accumulated in the foliage of grafted trees [30]. For instance, in Fino lemon trees, sour orange rootstock is considered a good Cl<sup>-</sup> and Na<sup>+</sup> excluder, whereas the *Citrus macrophylla* rootstock is a Cl<sup>-</sup> and Na<sup>+</sup> accumulator [39].

Citrus trees, under salinity stress, suffer growth reduction and some physiological and biochemical disturbances due to excessive concentrations of Cl<sup>-</sup> and Na<sup>+</sup> in leaves [37]. Depending on the soil type, irrigation method, and frequency, the soil solution salinity might also rise several fold between irrigations. Continual improvement of rootstocks and/or scions will be necessary to sustain irrigated citrus in increasingly salinized environments [30].

Sour orange is one of the most frequently used rootstocks in Mediterranean countries. It is known to be tolerant to salinity and calcareous soil among citrus rootstocks. However, it is highly susceptible to tristeza disease [37], that the disease should take into consideration the citriculture in Turkey and other countries which use sour orange as rootstock owing to be threatened by it. Genetically improved with favorable agronomical characteristics, such as resistance to pests and diseases such as the citrus tristeza virus, and salinity tolerance, rootstocks may be a long-term approach. Hence, screening studies based on physiological responses of genotypes to salinity stress should be used. In addition, continuous improvement of rootstocks is necessary to sustain cultivating citrus trees under salinized environments.

Yesiloglu et al. [40] established a screening study for the physiological evaluation of global tolerance to salinity rootstock collection of Çukurova University in the frame of the CIBEWU project, No: 015453. They screened 29 different genotypes that can be used as citrus rootstock under salinity stress assessed by several growth and physiological parameters such as fresh and dry weights of shoot and root; leaf chlorophyll concentration; and fluorescence, chloride, and Na content. High concentration of Cl and/or Na in the leaves of Citrus has been frequently related to disturbances in nutrition, gas exchange, and water relations. Unpublished data of the project regarding the genotypes used in the screening study and Cl<sup>-</sup> and Na<sup>+</sup> concentrations in root and leaves of the genotypes are presented below. 34-12 N citremon, 4475 SRA citrumelo, CRC 4475 Swingle citrumelo, *Severinia buxifolia* have accumulated higher chloride in leaves, while Tuzcu Cleopatra mandarin, Gou Tou, Rangpur lime, CRC 02 Volkameriana, Tuzcu 31-31 sour orange have lower chloride in leaves. Rubidoux trifoliata with trifoliata hybrids and *Severinia buxifolia* were poor Cl<sup>-</sup> excluder. It was found that Tuzcu Cleopatra mandarin and Rangpur lime are the best Cl<sup>-</sup> excluders. Gou Tou, Antalya Cleopatra mandarin have the lowest amount chloride in the roots, whereas *Citrus ichangensis*, Benecke trifoliata, Volkameriana, 08 A 3015 Rubidoux, and SRA Pomeroy have the highest values. According to the results, Tuzcu Cleopatra mandarin, Rangpur lime, Gou Tou, and Antalya Cleopatra mandarin were found to be the most tolerant to salt stress. *Severinia buxifolia*, CRC-4475 Swingle citrumelo, Local trifoliata, and Benecke trifoliata were the most sensitive group to salt stress. Data were evaluated by using modified "weighted-ranking" method based on the parameters of chlorophyll fluorescence ( $F_v'/F_m'$ ), leaf chlorophyll concentration by SPAD readings, leaf Cl and Na concentrations, leaf K and Ca concentrations, root K and Ca concentrations, growth parameters (shoot and root fresh and dry weights, shoot length, and leaf number), and visual ratings of leaf chlorosis. A classification which belongs to the screening study for salinity stress was performed as a result of the work and reported as follows in Table 1 (unpublished data). Genotypes were classified as very sensitive, sensitive, acceptable, tolerant, and very tolerant to salinity.

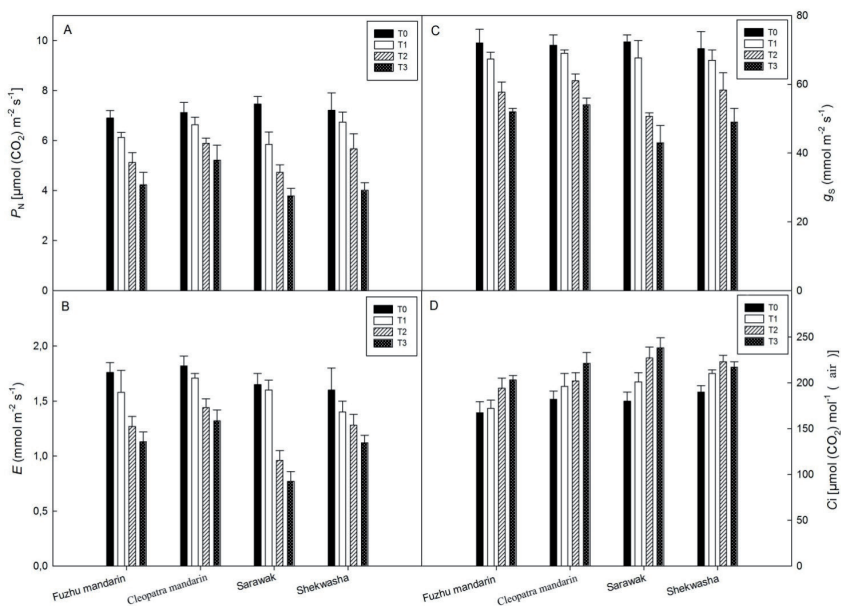
Khoshbakht et al. [41] reported that the effects of salinity on photosynthesis range from the restriction of CO<sub>2</sub> diffusion into the chloroplast, via limitations on stomatal opening mediated by shoot- and root-generated hormones, and on the mesophyll transport of CO<sub>2</sub>, to alterations in leaf photochemistry and carbon metabolism. The authors conducted a study and investigated the NaCl effects on gas exchange parameters of nine citrus rootstocks and reported that sour orange and Cleopatra mandarin were the rootstocks most tolerant to salinity of all the

Genotypes	Tolerance to salt stress
Rubidoux trifoliolate 08A 30.15	2
Citremom 34 12 N	1
Citrumelo 4475 SRA	1
AREC Swingle citrumelo	3
Antalya Cleopatra mandarin	3
Benecke trifoliolate	2
C-35 Citrange	4
Swingle citrumelo 4475 CRC	1
Volkameriana CRC 01	4
Volkameriana CRC 02	4
Citrumelo CRC 1452	3
Carrizo citrange	3
<i>Citrus ichangensis</i> CRC	3
<i>Citrus sulcata</i>	3
<i>Citrus sunki</i>	3
Macrophylla	2
Rangpur lime	5
Gou Tou sour orange SRA 506	4
Pomeroy trifoliolate SRA	1
Sacaton citrumelo	4
<i>Severinia buxifolia</i> SRA	1
Smooth Seville sour orange	4
Taiwanica	4
Troyer citrange	3
Tuzcu 31-31 sour orange	4
Tuzcu 891 sour orange	4
Tuzcu Cleopatra mandarin	5
Volkameriana	4
Local trifoliolate	2

\*1: very sensitive, 2: sensitive, 3: acceptable, 4: tolerant, 5: very tolerant

**Table 1.** Classification of rootstocks in collection of Çukurova University in respect of salinity tolerance

nine citrus rootstocks studied. Also, Cimen et al. [42] determined that the tolerances of Sarawak bintangor, Shekwasha, Fuzhu and Cleopatra mandarin to salt stress were determined by investigating the photosynthetic parameters and significant salinity effects on the photosynthetic performances of these rootstocks were reported (Figure 2).



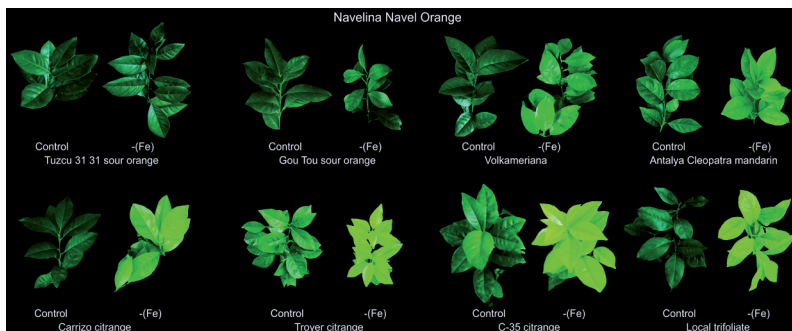
**Figure 2.** Effects of different salinity levels on photosynthetic rate (A), transpiration rate (B), stomatal conductance (C), and initial  $\text{CO}_2$  concentrations (D) of four genotypes. The bars indicate the standard deviation. (T0 = 0 mM NaCl, T1 = 50 mM NaCl, T2 = 75 mM NaCl, T4 = 100 mM NaCl). Data presented from Cimen et al. [42].

2.2. Alkalinity

It is well known that iron is an essential micronutrient for all higher plants including citrus. Most of the iron existing in rhizosphere cannot be taken up by plants because iron is highly insoluble. Two main working hypotheses have been put forward for this chlorosis. In the first working hypothesis, the main cause of Fe deficiency chlorosis is thought to be the inhibition of Fe acquisition by  $\text{HCO}_3^-$  in the rhizosphere. For the second hypothesis, Fe inactivation in the leaf apoplast by an alkalization process properly noted by the “distant effect” of  $\text{HCO}_3^-$  is thought to be the main trigger for Fe deficiency chlorosis in leaves [43]. The Mediterranean Basin is characterized by the prevalence of calcareous surface horizon. In these soils, iron (Fe) chlorosis can lead to diminished yields and even plant death, particularly in semiarid areas where irrigation water has high bicarbonate contents, soil pH is high (7.0–9.0), and organic matter content is low [44]. Citrus production is increasing throughout the Mediterranean countries and more and more citrus orchards are being planted on marginal soils. Mediterranean countries have a suitable climate for citrus production, but it is estimated that 20–50% of fruit trees grown in the Mediterranean basin suffer from iron (Fe) deficiency. The most prevalent cause of Fe deficiency in this region is the presence of high levels of carbonate ions in calcareous soils, characterized by a high pH [45]. These soils often have more than 20% of calcium and magnesium carbonates and are strongly buffered, with a pH between 7.5 and 8.5

[11]. Fe uptake is highly dependent on soil pH, and Fe activity in solution decreases 1000-fold for each pH unit rise to reach a minimum within the range from 7.4 to 8.5 [46]. Leaf Fe chlorosis in plants is an old problem occurring in areas of calcareous and/or alkaline soils. Yield reductions from Fe-induced leaf chlorosis have been found in tomato, raspberry, kiwifruit, pineapple, vines, and citrus [47]. Moreover, the severity of leaf chlorosis and the differential behavior of genotypes can be determined by the chlorophyll concentration in leaves [11, 46, 48]. The high level of bicarbonate ions in the soil affects metabolic processes in roots and leaves, decreasing soil and plant Fe availability, leading to the condition known as lime-induced iron chlorosis. The most evident effect of Fe chlorosis is a decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls and producing yellow, chlorotic leaves. The loss of pigmentation is caused by decreased chlorophyll content in chloroplasts. This negatively affects the rate of photosynthesis and, therefore, the development of biomass. Fe deficiency affects the physiology and biochemistry of the whole plant, as Fe is an important cofactor of many enzymes, including those involved in the biosynthetic pathway of chlorophylls [10, 11].

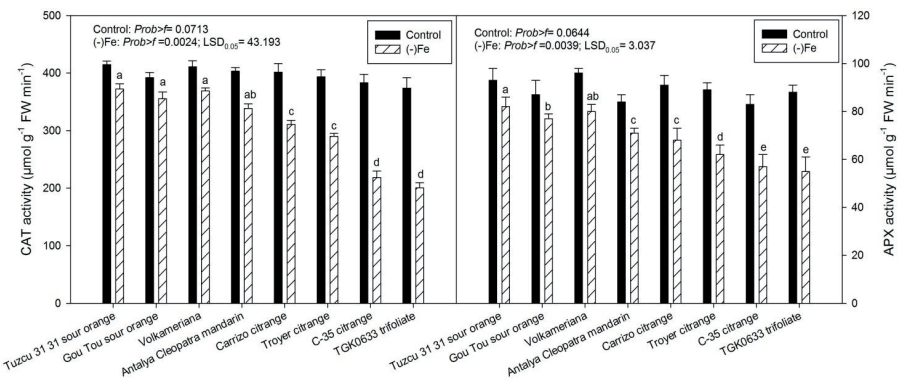
The use of rootstocks in fruit production includes not only a stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metal, nutrient stress, water stress, and alkalinity [7]. Recent studies showed that citrus rootstocks had different tolerance levels to iron deficiency [48, 49]. Studies emphasized that high pH conditions reduced iron uptake in citrus rootstocks [48, 49, 50, 51, 49]. Also, rootstocks affect tree growth, fruit quality, and yield [15, 48, 52]. Moreover, scion behavior depends in part on the rootstock-induced effects on leaf gas exchange [53]. González-Mas et al. [53] indicated that in calcareous soils, citrus production depends on the availability of suitable rootstocks that are tolerant to low Fe soil conditions. Studies have found that "Volkameriana," and "sour orange" plants were tolerant; "Carrizo and Troyer" citrangs were intermediate, whereas the "*Poncirus trifoliata*" rootstock was more sensitive to iron chlorosis [46, 48, 49, 51, 54]. In addition, Cimen et al. [55] indicated that Young "Navelina" orange trees budded on Tuzcu 31-31 sour orange, and Gou Tou sour oranges performed best under Fe deprived conditions in plant growth chamber. Navelina on Volkameriana and Cleopatra mandarin was moderate; C-35 citrange and local trifoliolate were poorly adapted to lime-induced Fe deficiency (Figure 3).



**Figure 3.** Response of Navelina Nave orange budded on eight different rootstocks in response to iron deficiency. Data presented from Cimen et al. [55].



Among physiological processes, photosynthesis is the basic determinant of plant growth and productivity, and the ability to maintain the rate of carbon assimilation under environmental stress is of fundamental importance to plant production [56]. Since Fe catalyzes chlorophyll biosynthesis [57], it would be expected to promote the photosynthetic rate ( $P_n$ ) while Fe deficiency to reduce it [58, 59]. Most of the knowledge concerning the effect of Fe deficiency on the photosynthetic parameters has been obtained with annual plants. However, relatively few studies have focused on the consequences of induced Fe deficiency on photosynthesis in evergreen fruit trees and especially in citrus. On the contrary, Fe is a component of several metalloenzymes, including peroxidase and catalase. Although both enzymes could be used as biochemical indicators of Fe availability in citrus [36], there are contradictory reports concerning the effect of Fe deficiency on catalase and peroxidase activity [60]. Cimen et al. [55] reported that at sufficient Fe supply, plants had higher activity of catalase (CAT) than the plants with Fe deprived conditions. Slight decreases were recorded on the Navelina orange leaves of Tuzcu 31-31 and Gou Tou sour oranges, while decreases were remarkable in the leaves of C-35 citrange and TKG0633 (obtained by a selection of trifoliate orange in Turkey) under short supply of Fe. In addition, Navelina leaves of C-35 citrange and TKG0633 displayed maximum decreases in APX (ascorbate peroxidase) activity, similarly CAT activity. There were no significant APX activity decreases in the leaves of Tuzcu 31 31, Gou Tou sour oranges, Volkameriana, and Antalya Cleopatra mandarin (Figure 4).



**Figure 4.** Catalase and ascorbate peroxidase activities of Navelina leaves of different rootstocks under Fe sufficient and deprived conditions. The bars show the standard deviation. Data presented from Cimen et al. [55].

The intensity of iron chlorosis can be quantified by total Fe, active Fe, leaf chlorophyll meter, photosynthetic parameters, enzymes, plant growth parameters, and visual ratings of leaf chlorosis. One of the distinctive characteristics of iron deficiency in field crops is the lack of correlation between leaf iron content and chlorosis. This has been termed the "chlorosis paradox." Therefore, leaf chlorophyll contents are generally used to monitor iron chlorosis [61]. The use of visual ratings and readings of a portable chlorophyll meter are the most efficient approaches to define iron chlorosis in citrus [51]. Yesiloglu et al. [40] established a screening



study for the physiological evaluation of global tolerance to lime-induced Fe chlorosis in rootstock collection of Çukurova University in the frame of the project CIBEWU, No: 015453 and evaluated by using modified "weighted-ranking" method based on the parameters of total Fe, active Fe, chlorophyll fluorescence ( $F_v'/F_m'$ ), leaf chlorophyll concentration by SPAD readings, visual ratings of leaf chlorosis, and shoot and root weight.

Local trifoliolate, Rubidoux trifoliolate, and Benecke trifoliolate exhibited severe chlorosis and were more chlorotic than other genotypes. Cleopatra mandarins among mandarin and its hybrids were more tolerant than Sunki and Calamondins. Macrophylla was the best in lemon and lemon hybrids group. Campbell [50] reported that Macrophylla was well adapted to soils of high pH. Volkameriana and Rangpur were almost the same. Gou Tou was really so tolerant to iron deficiency. All sour oranges were similar to each other. Alanya Dilimli sweet orange is a variety selected in south of Turkey. It is known to be very resistant to high pH conditions. The results confirm that Alanya Dilimli is very tolerant to high pH. According to the results, Carrizo citrange, Flhorag1, Macrophylla, Antalya Cleopatra mandarin, Tuzcu 31-31 sour orange, Gou Tou sour orange, and Alanya Dilimli sweet orange were more tolerant than others in citrus rootstock collection of Çukurova University (Table 2 – unpublished data).

Genotypes	Tolerance to iron chlorosis
Rubidoux trifoliolate 08A 30.15	2
Citremon 34 12 N	3
Citrumelo 4475 SRA	3
Arec Rubidoux trifoliolate	3
Arec Swingle citrumelo	2
Benecke trifoliolate	2
Swingle citrumelo 4475 CRC	2
Carrizo citrange	4
Flhorag1	5
Pomeroy trifoliolate SRA	2
Sacaton citrumelo I	2
Sacaton citrumelo	2
Citrumelo 1452 CRC	2
Local trifoliolate	1
Troyer citrange	3
Antalya Cleopatra mandarin	4
<i>Citrus sunki</i>	4
Calamondin CRC	2

Genotypes	Tolerance to iron chlorosis
Calamondin 108 USDA	1
Tuzcu Cleopatra mandarin	2
Volkameriana CRC 01	3
Volkameriana CRC 02	3
Macrophylla	4
Rangpur lime	3
Volkameriana	3
<i>Citrus ichangensis</i> CRC	3
<i>Citrus sulcata</i>	2
<i>Severinia buxifolia</i> SRA	2
Alanya Dilimli sweet orange	5
Gou Tou sour orange SRA 506	5
Smooth Seville sour orange	3
Taiwanica	2
Tuzcu 31-31 sour orange	5
Tuzcu 891 sour orange	4

\*1: very sensitive, 2: sensitive, 3: acceptable, 4: tolerant, 5: very tolerant

**Table 2.** Classification of the rootstocks in collection of Çukurova University in terms of tolerance of iron deficiency.

Although trifoliate orange has many advantages in terms of tolerance to abiotic and biotic stresses, it is susceptible to calcareous soil conditions. However, there are some superior genotypes to improve the tolerance to high pH of present rootstocks by hybridization and crossing [44].

Several rootstock breeding programs have been carried out by different countries leading in citriculture. Forner et al. [62] reported two new citrus rootstocks named F-A 5 and F-A 13, released in Spain. These rootstocks are hybrids of Cleopatra mandarin × Rubidoux trifoliate crosses with a high level of tolerance to lime-induced iron chlorosis. Besides, Bowman and Rouse [63] mentioned a new citrus rootstock named as US-812 which is a hybrid obtained by a cross between Sunki mandarin (*Citrus reticulata*) and Benecke trifoliate orange in USDA Indio Research Station, California. They have reported that using Valencia trees budded on to US-812 resulted in some tolerance to high alkalinity under pH conditions 8.1– 8.3. Moreover, Federici et al. [64] indicated that three citrus rootstocks released in August 2009 by the University of California named as “Bitters,” “Carpenter,” and “Furr” trifoliate hybrids, tested as C22, C54, and C57, respectively, by crossing Sunki mandarin × Swingle trifoliate orange. Bitters were found to be very tolerant to calcareous soil, whereas Carpenter and Furr were found to be moderately tolerant.

### 2.3. Drought

Mediterranean region has a subtropical climate and is an important region for citriculture. A significant amount of high-quality citrus fruits is produced in Mediterranean countries such as Spain, Turkey, Italy, Greece, Egypt, Morocco, and Tunisia. Fruit yield and quality are affected by genetic traits together with environmental factors. Fruit crops are frequently exposed to environmental stresses spontaneously or by conventional agronomic conditions. Some of these conditions such as high temperature may last only for a short period of time, whereas lack of water in soil may last for longer periods. Global warming is a type of greenhouse effect which is defined as the increase of Earth's average surface temperature due to the effect of greenhouse gases, such as carbon dioxide emissions from burning fossil fuels or from deforestation, which trap heat that would otherwise escape from Earth.

Yaacoubi et al. [65] indicated that Mediterranean fruit tree production is facing major changes that have environmental and socioeconomic consequences. Climatic changes related to temperature warming have been reported worldwide.

Drought stress, as one of the most ominous abiotic factors limiting the productivity of horticultural crops, is increasingly growing in dimension of severity in many regions of the world [66]. In general, the mechanism of drought resistance in plants can be explained as drought escape, drought avoidance, and drought tolerance. These traits consist of osmotic adjustments, cell membrane stability, epicuticular wax, partitioning and stem reserve mobilization, manipulation and stability of flowering processes, and seedling drought traits.

Drought tolerance is a complex trait that is important at different growth stages and involves multiple adaptations. Fundamental to this is the ability to maximize the extraction of water from the soil while minimizing loss from the leaves. Morphological adaptations include the development of deep roots and alterations in leaf morphology and cuticle structure, while physiological adaptations involve changes in stomatal density to maximize water uptake and retention [67, 68, 69].

Drought stress effects on the plant may range from slight suppression of growth and yield to temporary wilting, in which leaves flag but recover after transpiration demands decrease, to permanent wilting in which the plant suffers injury and death [70]. A plant responds to a lack of water by halting growth and reducing photosynthesis and other plant processes in order to reduce water use. As water loss progresses, leaves of some species may appear to change color, usually to blue-green. Foliage begins to wilt and, if the plant is not irrigated, leaves will fall off and the plant will eventually die. Drought lowers the water potential of a plant's root and upon extended exposure, abscisic acid is accumulated, and as a result stomatal closure occurs. This reduces a plant's leaf relative water content. The time required for drought stress to occur depends on the water-holding capacity of the soil, environmental conditions, stage of plant growth, and plant species [71]. Plants growing in sandy soils with low water-holding capacity are more susceptible to drought stress than plants growing in clay soils. A limited root system will accelerate the rate at which drought stress develops. A plant with a large mass of leaves in relation to the root system is prone to drought stress because the leaves may lose water faster than the roots can supply it. The root system has a great importance when the plant faces

drought. For instance, Rough lemon rootstocks are very drought tolerant because of their extensive, deep root systems. Newly installed plants and poorly established plants may be especially susceptible to drought stress because of the limited root system or the large mass of stems and leaves in comparison to roots.

Citrus, a perennial crop with a long orchard life, is likewise a globally important fruit crop responsible for world trade and often exposed to the vagaries of soil and atmospheric drought stress [72]. Drought stress is known to restrict the vegetative growth and yield of citrus, in addition to adversely affecting fruit quality and incurring huge economic loss to the citrus growers [73]. Therefore, screening and selection of germplasm are of great importance in terms of drought tolerance.

Pedrosoa et al. [74] reported that citrus rootstocks have differential capacities for supplying shoot tissues with water and carbon, improving the resistance to biotic and abiotic stresses and affecting plant water status and photosynthesis. Water relations have been well studied in citrus trees, showing that rootstocks alter the physiological performance under water deficit through variations in plant hydraulic conductance, leaf water potential, and stomatal conductance [75, 76, 77]. In addition, several studies have found that citrus rootstocks showed different performances when they are exposed to drought [13, 73].

Treeby et al. [78] investigated irrigation management and rootstock effects on navel orange and reported that irrigation management is far more critical for external fruit quality for trees on sweet orange and, to a lesser extent, trees on the citranges compared to trees on trifoliolate orange and Cleopatra mandarin.

Some studies also indicate that using tetraploid rootstocks increases the drought tolerance in comparison to their diploid clones in citrus. Allario et al. [79] reported that polyploidy is common in many plant species and often leads to better adaptation to adverse environmental conditions. The authors examined the drought tolerance in diploid (2x) and autotetraploid (4x) clones of Rangpur lime (*Citrus limonia*) rootstocks grafted with 2x Valencia Delta sweet orange (*Citrus sinensis*) scions, named V/2xRL and V/4xRL, respectively. The results of the authors showed that using tetraploid clones of Rangpur lime had increased the drought tolerance in grafted sweet orange.

### 3. Germplasm and genetic variability

The origin of citrus is believed to be southeast Asia, including south China, northeastern India, and Burma. Commercial citrus species and related genera belong to the order Geraniales, family Rutaceae, and subfamily Aurantoidea. Fruit crops is a very heterogeneous group of plants including trees, shrubs, climbing vines, and perennial herbs. They inhabit different climates, ranging from tropical to subarctic zones and altitudes from sea level to higher mountains. Fruit crops also differ considerably with respect to their origin, taxonomy, and breeding systems. The biodiversity in plants has been progressed by natural and artificial hybridization and mutation which are the basic resources of biological evolution. The deteri-

oration of ecosystem directly or indirectly by human beings has always been causing the destruction of biodiversity and many advanced genotypes [80].

There is extensive genetic diversity in citrus. Aydin and Yesiloglu [81] reported that the genus *Citrus* L. belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family [82]. The Aurantioideae is one of seven subfamilies of Rutaceae which consists of two tribes and 33 genera. Each of tribes Clauseneae and Citreae is composed of three subtribes. Clauseneae includes Micromelinae, Clauseninae, and Merrillinae, and Citreae has Triphasiinae, Citrinae, and Balsamocitrinae. The Citrinae is distinct from all the other subtribes in the subfamily by having pulp vesicles in the fruit. This subtribe contains three groups: primitive citrus fruit, near citrus fruit, and true citrus fruit trees. True citrus fruits have six genera: Clymenia, Eremocitrus, Microcitrus, Poncirus, Fortunella, and Citrus [83].

All rootstocks and varieties used are included in the genus Citrus, except for kumquats (*Fortunella* spp.) and trifoliolate orange (*Poncirus trifoliata* L. Raf.), the latter is used exclusively as a rootstock. Trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] is an important citrus relative for breeding new rootstocks. In addition to its tolerance to citrus tristeza virus, citrus nematodes, and cold weather and edaphic conditions, the dominant nature of the "trifoliolate" leaves of trifoliolate orange is a useful morphological marker in the visual identification of hybrids from crosses using trifoliolate orange as male parent. Some hybrids of commercial interest, including citranges (sweet orange × trifoliolate orange) and citrumelos (grapefruit × trifoliolate orange), are used as rootstocks.

In general, the diversity of genetic structures in the subfamily of Aurantioideae, which occurred within the steps of biological evolution, is the genetic resource that has been lost before the determination of their characteristics during the rapidly vanishing process. Preservation of this kind of material is a challenge particularly for "conservation breeding" branch of plant breeding [80]. The determination of citrus genus and close relative genus and species collections with *in situ* and *ex situ* structure in the selected countries and regions, describing and establishing international legal tender for them, are very important.

The richness of germplasm has benefited genetics and breeding research in the countries that have a long history of citriculture. China is the most important place of origin for citrus. The long history and diversified climates enable China to harbor the most citrus varieties. Southern China is one of the centers of diversity for *Citrus* and related genera such as *Fortunella*. A National Citrus Germplasm Repository was established in China in the early 1980s, and a record in 1996 reported 1041 accessions [84, 85]. In India, there is an *in situ* germplasm including 627 accessions as it was reported by Singh [86], and eight *ex situ* citrus conservation collections have been established. Three *in situ* collections were established in Malaysia. In addition, there are three collections in Indonesia and Thailand, two collections in Philippines. The orchards of The Federal Fruit Crops Research Station in Tsukuba, Okitsu, and Kuchinotsu have the widest diversity collection of citrus and the relative types. It is declared that there are totally more than 1300 accessions in these three stations [84]. The original natural dispersion areas of Microcitrus and Eremocitrus as well as many relative types of citrus are located in Australia and the main collection in Australia is located at the Biological and Chemical Research Institute under the NSW Agriculture & Fisheries Depart-

ment in Rydalmere in New South Wales [84]. Tuzcu [80] reported that the collection of citrus in California began in the 1890s, and the conservation and utilization system of these were structured in 1910, shortly after the establishment of Citrus Experiment Station (CES) in Riverside [87]. H.J. Webber, the first manager of the station, ensured the establishment of Citrus Variety Collection in 1917 in the field which is currently in the Riverside Campus Area of California University. United States Department of Agriculture (USDA) National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) was established as a top organization for the coordination of citrus genetic resources studies in 1974. It is working in close cooperation with other two establishments. There are about 350 virus-free accessions under its conservation [84]. The most important collection is located at National Research Center for Cassava and Fruit Crops, CNPMF in Cruz das Almas/Bahia, which includes 1858 accessions in Brazil. Valencia Agricultural Research Institute [Instituto Valenciano de Investigaciones Agrarias (IVIA)] is responsible for all the actions regarding citrus genetic resources. According to the last records, there are 478 elite accessions in total including 13 genera (16 accessions) of the Aurantioidea subfamily in addition to Citrus genus in IVIA [84, 88]. In France, 1300 accessions at Agricultural Research Station SRA tied to National Agricultural Research Institute (Institut National de la Recherches Agronomiques – The National Agronomic Research Institute (INRA) at San Nicola in Corsica Island exist in “SRA Citrus Collection” [84]. In terms of citrus genetic resources, mainly, there is one established citrus germplasm in Turkey. This establishment is named as Tuzcu Citrus Collection (TCC) and consists of 964 accessions in Çukurova University Faculty of Agriculture [80].

#### 4. Some developed citrus rootstocks and their tolerance to abiotic stress

The first use of rootstocks in citriculture was in 1842 against Phytophthora in Azores Islands through the use of resistant rootstocks. Since then, commercial citrus orchards are established by combining scion and rootstocks in order to achieve the highest quality for the scion. The use of rootstocks in citrus decreases the long juvenility period and allows the cultivation of citrus under several abiotic and biotic stress conditions. The physiology of the whole tree is affected by rootstock, including traits of economic relevance such as fruit yield, fruit size, juice quality, tree vigor, and resistance against biotic and abiotic stresses [89]. Fruit maturation, fruit holding on tree, and postharvest preservation are also affected by rootstock [90].

There is no ideal rootstock in order to manage all abiotic and biotic stress conditions. For instance, sour orange (*Citrus aurantium* L.) which has a high adaptation capability to different soil conditions has been the most commonly used rootstock in commercial citrus trees. Also, sour orange (*Citrus aurantium* L.) is tolerant to root rot, citrus blight disease, calcareous soils, water deficit, and cold, inducing high yield and high fruit quality. However, sour orange is susceptible to citrus tristeza virus, and the usage of this rootstock is decreasing in some countries due to the existence of CTV. On the contrary, Rangpur lime (*C. limonia*), Volkamer lemon (*C. volkameriana* V. Ten. and Pasq.), and rough lemon (*C. jambhiri* Lush.) are drought resistant and increase the fruit yield of the grafted scion. Besides, they reduce the fruit quality

as compared to fruits obtained from sour orange, trifoliate orange, Carrizo citrange, and Troyer citrange. However, they are sensitive to cold.

The search for new rootstock in citrus production as well as many fruit species is necessary to sustain production under the inevitable abiotic stresses in many different ecological conditions of the citrus-growing areas of the world. Also, new diseases, the spread of the known diseases, and different environmental conditions affected by the climatic change force the demand for developing new citrus rootstocks. Below, we have tried to cover and explain some of the newly released citrus rootstocks obtained by breeding programs carried out in several countries by several researchers.

Swingle citrumelo is a hybrid of Duncan grapefruit and trifoliate orange produced in 1907 by Swingle and released by the United States Department of Agriculture in 1974. Since then, it has been used successfully as a rootstock in many countries. Most varieties produce very satisfactory yields of good to excellent quality fruit on Swingle citrumelo. Fruits produced on Swingle citrumelo are similar to sour orange, Carrizo, and Troyer citranges in terms of fruit quality. The trees have good cold-hardiness only slightly inferior to that of trees on trifoliate oranges. Scions on Swingle citrumelo are very tolerant to CTV, blight, and root rot as well as being resistant to citrus nematodes. They also have good exocortis and xyloporosis tolerance. The trees grow well on most soils and are reportedly especially tolerant of waterlogged conditions. In contrast, Swingle citrumelo is an unacceptable choice in heavy clay, calcareous soils, and high pH soils. Swingle is sensitive to high pH soils and is unsuitable for highly calcareous soils. Therefore, it is not so popular in many Mediterranean countries. Tolerant to environmental and soil conditions, Swingle citrumelo is sensitive to high chloride levels in soil and irrigation water but is more salt tolerant than other trifoliate hybrids such as Carrizo and Troyer citranges. Swingle has moderate drought tolerance [91].

Citranges are known as hybrids of sweet orange and trifoliate orange. The main purpose of the citrange development in Florida was to combine good traits of sweet orange with the cold-hardiness of the trifoliate orange in order to create cold hardy scions. Although unsuccessful, a most significant source of new rootstocks was produced instead. There are several named selections, the more important of which are Carrizo and Troyer citranges. These are hybrids of Washington navel orange and *Poncirus trifoliata*. The original crosses were made in the early 1900s by the United States Department of Agriculture with the intention of producing cold-tolerant scion varieties. They were later identified as being suitable for use as rootstocks. Fruit quality of the scion on Carrizo and Troyer is excellent. Trees on both grow moderately vigorous on a range of soil types but have poor salt tolerance and are sensitive to calcareous soils and exocortis virus. They have intermediate frost tolerance but are less cold-hardy than those on Cleopatra mandarin and trifoliate orange. In Turkey, Troyer citrange and trifoliate orange show superior fruit quality for Satsuma mandarins, and they are the mainly used rootstock in Aegean region of Turkey. However, using Carrizo citrange as a rootstock to especially mandarins and oranges is more common due to high soil pH levels in the Mediterranean region of Turkey. In general, the performance of Carrizo citrange is slightly better than Troyer in cancerous soils of Çukurova. However, sour orange is the main rootstock in Turkey. Ninety percent of citrus varieties in Turkey are grafted on the sour orange rootstock.

Benton citrange is a hybrid of Ruby Blood orange and trifoliate orange. It was bred in the late 1940s by the Department of Agriculture, New South Wales, Australia. The seed was first released to the industry in 1984. There are some commercial plantings using this rootstock which were established in 1990, but poor seed production in the seed source trees has been an impediment to its widespread usage. Because of its erratic performance under orange and mandarin scions, it is only recommended for Eureka lemons in Australia and only replant situations in Queensland. In Florida, Benton citrange is recommended for small-scale commercial trials with oranges and grapefruit. Trees on Benton rootstock are reported to be moderately cold tolerant and higher yielding.

C-35 citrange was bred by the University of California and released in 1987 and is a hybrid obtained by crossing Ruby Blood orange  $\times$  Webber-Fawcett trifoliate. C-35 is tolerant to *Phytophthora* and CTV and resistant to citrus nematodes. Frost tolerance is good as or slightly better than Carrizo. Trees grow 25% smaller than Carrizo, making C-35 a candidate for closer spacing plantings. Trees grown in sandy, loam, and clay soils are satisfactory, but they are more sensitive to calcareous soils than Carrizo. C-32 has the same parentage as C-35 citrange and is a hybrid between Ruby orange and Webber-Fawcett trifoliate. Its very low seed production makes this citrange's seedling propagation difficult in order to use as rootstock [91].

Several rootstock breeding programs have been carried out by different leading countries in citrus industry in order to handle increasing problematic issues by abiotic and biotic stress factors.

Forner et al. [62] reported two new rootstocks released in Spain. Forner-Alcaide 5 (F-A 5) and Forner-Alcaide 13 (F-A 13) are two interspecific hybrids obtained through traditional hybridization by a senior author in a program for breeding citrus rootstocks at the IVIA in Moncada (Valencia), Spain. The researchers aimed to obtain new rootstocks tolerant to CTV, salinity, and lime-induced chlorosis and resistant to *Phytophthora*. They reported the resistance of F-A 5 and F-A 13 to CTV. In addition, F-A 5 was found to be more tolerant to lime-induced chlorosis than Carrizo citrange, whereas F-A 13 is less tolerant [92]. Besides, both rootstocks have good tolerance to salinity and an excellent tolerance to flooding, as reported. Gonzalez-Mas et al. [53] conducted a rootstock field study in order to investigate rootstock effects on leaf photosynthesis in "Navelina" trees grown in calcareous soil. Authors have used seven new citrus rootstocks with Carrizo citrange obtained by J. Forner at the IVIA: F-A 5, F-A 13, F-A 418, F-A 517, 030116 (Cleopatra mandarin  $\times$  *P. trifoliata*), 020324 (Troyer citrange  $\times$  Cleopatra mandarin), and 230164 (*C. volkameriana* Ten. and Pasq.  $\times$  *P. trifoliata*). Trees grafted on F-A 5 performed best under these calcareous soil conditions, whereas those on Carrizo citrange were poorly adapted, regarding the parameters investigated.

Bowman and Rouse [63] reported the release of US-812 citrus rootstock in May 2001 by the Agricultural Research Service of the USDA and is the result of a cross between Sunki mandarin and Benecke trifoliate. The rootstock was found to be highly productive of good quality fruit with a moderate vigor (standard medium tree size) as it was reported. The US-812 shows tolerance or resistance to CTV and citrus blight. It was reported that US-812 has good soil adaptability and disease resistance. Valencia orange grafted on US-812 performed well under high pH conditions in calcareous soils. Bowman [93] also introduced US-802 and US-897 and



reported high productive per tree size, good soil adaptability and disease resistance, tolerance of Diaprepes and Phytophthora complex, large contrast in vigor, and tree size as the forthcoming features of the rootstocks. Besides, US-942 rootstock is very highly productive with good fruit quality and soil adaptability.

According to the report of Federici et al. [64], three new citrus rootstocks were released in 2009 by the University of California, Riverside. These three rootstocks were named as “Bitters,” “Carpenter,” and “Furr” trifoliate hybrids, tested as C22, C54, and C57, respectively, and obtained by sexual hybridization of Sunki mandarin × Swingle trifoliate orange. The main character of these three hybrids is they all show good tolerance to citrus tristeza virus.

As reported by Federici et al. [64], “Bitters” showed good tolerance to freezing. It is tolerant to CTV, moderately tolerant to *Phytophthora parasitica*, not very tolerant of citrus nematode, and very tolerant of calcareous soil. “Carpenter” showed moderate tolerance to freezing. It is tolerant to CTV, moderately tolerant to *P. parasitica*, very tolerant of citrus nematode, and moderately tolerant of calcareous soil. “Furr” also showed good tolerance to freezing, and it is tolerant to CTV, very tolerant to *P. parasitica*, very tolerant of citrus nematode, and moderately tolerant of calcareous soil [64].

## 5. Breeding techniques for tolerance to abiotic stress: Traditional and biotechnological approaches

Nearly all commercial citrus in the world are grown as grafted trees, with the scion cultivar budded on a selected rootstock cultivar. A good scion and rootstock combination supports the development of trees that bear large quantities of high-quality fruit. However, many available rootstocks are inadequate to meet the emerging needs and challenges. A large proportion of the problems faced by the citrus industry could be overcome by the use of improved rootstocks [93, 94]. However, developing an improved rootstock is a long-term approach because of the several difficulties, mainly the complexity of citrus biology. Typically, it takes at least 15 years from the beginning of a cross-hybridization program until a new selected rootstock is released to the industry for a commercial use.

### 5.1. Traditional breeding

Although somatic hybridizations via *in vitro* culture methods and genetic transformation via the regeneration process of plant tissues have opened new enthusiastic prospects for citrus genetic improvement, classical breeding techniques still remain important for citrus breeding. Genetic variations have a great importance in terms of plant breeding. These variations can be obtained either spontaneously or artificially by mutations and sexual hybridizations in order to achieve specific breeding objectives. Planned or unplanned sexual hybridizations have been responsible for the evolution of the new genotypes for using either rootstock or scion. Many intergenetic hybrids were produced by controlled pollination. For example, citranges (*C. sinensis* × *P. trifoliata*), citrumelos (*C. paradisi* × *P. trifoliata*), citremons (*C. lemon* × *P. trifoliata*),

citradia (*C. aurantium* × *P. trifoliata*), citrusquat (*Fortunella* spp. × *P. trifoliata*), and Eremoradia (*Eremocitrus glauca* × *C. aurantium*).

Citrus flowers usually bloom in the spring in one great flush, except acid limes and lemons which are noted for flowering throughout the year in cold subtropical climates. In tropics, flowering may occur more than once throughout the year. In addition, drought and excessive fertilizing may induce flowering. Citrus flowers are mostly hermaphrodite and release pollen when the stigma is receptive. However, there are some exceptions such as staminate and pistillate flowers occurring in lemons Satsumas, Shamouti, and sour orange. Besides, late harvest of the fruits instead of optimum harvest time and fertilization deficiencies can increase the ratio of staminate and pistillate flowers on trees. Besides, W. Navels are known to have the ability to set parthenocarpic fruits due to their pollenless flowers. The flowers never close; the petals merely shed a few days later. The stigma becomes receptive just before the bud breaks open, but the stamens usually do not release pollen until several hours later, after the flower is fully open. This should be considered in terms of collecting flowers for pollens to be used as male parents. Most pollination in citrus is done by insects except for varieties showing parthenocarpy that no pollination is required for fruit development.

Many citrus cultivars are known to be self-incompatible and, in some cases, cross-incompatible. With such cultivars, an appropriate pollen supply and pollinating agents is needed. Pollination requirements vary among the species and cultivars. For example, open pollinated flowers of grapefruit result in significant increases in both fruit number and seed numbers. When lemons are protected from insect visitations, a set of fruits decreases. Pummelos are known to be self-incompatible as well as Clementines, Lee, Page, Nova, and Robinson. In contrast, no pollination problems have been observed in citron, kumquat, Meyer lemon, and trifoliata orange, but there have been problems of seed set in "Morton" and "Troyer" citrange [95].

We consider the apomixes as one of the major problems in citrus rootstock breeding. Nucellar embryony is the most unusual feature that exists in the reproductive biology of citrus. This mechanism limits crossing and selfing in many varieties. Most of the genotypes that can play important roles as female parents in traditional crossing studies are highly apomictic (for example, see Table 3, unpublished data recorded at the Çukurova University, Faculty of Agriculture, Department of Horticulture, Citrus Germplasm Orchards). Hence, citrus breeding is limited by nucellar embryony of most diploid genotypes [96, 97]. The nucellar tissue which surrounds the megagametophyte can produce additional embryos (polyembryony) which are genetically identical to the parent plant. In contrast, zygotic seedlings are sexually produced and inherit genetic material from both parents. Zygotic and nucellar embryos can occur in the same seed. Not all citrus species exhibit the characteristic of polyembryony, a some produce only zygotic embryos (pummelo, citron, Clementine, Temple, and Persian lime, for example). Others produce only nucellar embryos. Many citron and lime varieties produce a significant percentage of zygotic seedlings but oranges, grapefruit, and many mandarins usually have a low percentage.

Genotype	Polyembryony (%)
Tuzcu 01-21 sour orange	60
Carrizo citrange	92
Troyer citrange	71
Swingle citrumelo	50
Local trifoliolate ( <i>P.trifoliata</i> )	80
Cleopatra mandarin	95
Volkameriana	60
Rough lemon	56
Florida Rough lemon	93
Sunki mandarin	50
Yuzu	60
Taiwanica	50
King mandarin	81
<i>C. myrtifolia</i>	85
<i>C. obovidea</i>	76

**Table 3.** Polyembryony ratio of some potential genotypes in CU Citrus Germplasm Orchards to be used in breeding studies.

In addition, sexual hybridization faces some constrains in citrus, due to high heterozygosity, long juvenility, and polyembryony of most citrus cultivars. Moreover, it is difficult to identify sexual hybrid embryos in their early stage. In this case, using trifoliolate oranges, which are valuable rootstocks due to their characteristics such as cold-hardiness and resistance to root rot, CTV, and nematodes, gains another importance for citrus rootstock breeding against polyembryony. Since the trifoliolate character is dominant, progenies exhibiting the trifoliolate phenotype of the pollen parent can be considered as putative hybrids. So using trifoliolate trait as a morphological marker is useful for early separation and characterization in citrus rootstock breeding studies. In contrast, progenies obtained by crossing combinations using polyembryonic genotypes as females have to be identified via molecular markers systems (RAPD and SSR) in order to speed up the separation of zygotic hybrids from nucellar seedlings.

Controlled cross-pollination in citrus is mainly performed for combing desirable traits from different genotypes or species and inducing heterosis. Based on this method, many hybrid rootstocks between citrus and *Poncirus* have been developed (see Section 4).

Controlled pollination is relatively easy in citrus. Seed parent and pollen parent flowers should be protected against contamination. Emasculation is generally easy and less effortless at the flowers that are nearly ready to open. Emasculation is accomplished by gently separating the petals, pulling off the anthers while avoiding contact with stigma. Pollination should be carried

out immediately after emasculation. A special storage of the pollen is seldom necessary while crossing within the genus *Citrus*. The genera *Poncirus* and *Fortunella* can be crossed with *Citrus*. Trifoliate orange naturally bloom earlier than citrus, so the pollens must be stored until the flowering time of *Citrus*. Pollens should be collected from unopened flowers from the branches of trees. After a waiting period of 24 h in the room temperature, a high quantity of pollen grains can be collected from anthers. Calcium chloride can be used as a drying agent just before storage of the pollen grains in a cold condition. On the contrary, *Fortunella* bloom much later than citrus in many areas. Figure 5 presents a traditional breeding procedure in citrus at Çukurova University, Citrus Germplasm Orchards.



**Figure 5.** Traditional cross-hybridization in citrus. (A) a large unopened bud, (B) emasculation, (C) pollination of the emasculated flower, (D) cotton pad wrapping around the twig, (E) bagged twig, (F) general view of the seed parent after crossing.

Fruit breeding, especially using classical breeding methods, is a difficult work taking a lot of time. In terms of citrus, chance seedlings were the main source for the cultivars, and spontaneous mutations on branches were used to select new cultivars. Current breeding projects in the present day is crossing superior selections and inducing mutations for seedlessness as well as crossing at different ploidy levels for seedless triploids.

## 5.2. Current biotechnologies applied in rootstock breeding

Genetic improvement of citrus through conventional breeding is limited by their genetic and reproductive characteristics. Citrus species have a complex reproductive biology, with many cases of cross- and self-incompatibility, apomixis, and high heterozygosity, and most of them have very long juvenile periods. Most species are highly heterozygous and produce progeny segregating widely many characters when crosses are made. In addition, juvenile periods are often extensive and most significantly, the presence of adventitious embryos in the nucellus of developing ovules of most citrus types greatly inhibits hybrid production [97, 98].

Plant somatic hybridization via protoplast fusion has become an important tool in plant improvement, allowing researchers to combine somatic cells (whole or partial) from different cultivars, species, or genera resulting in novel genetic combinations including symmetric allotetraploid somatic hybrids, asymmetric somatic hybrids, or somatic cybrids [99].

Briefly, the development of hybrid plants through the fusion of somatic protoplast derived from different sources of two different plant cultivars, species, and genera is called somatic hybridization. The technique of somatic hybridization involves the following steps: (1) isolation of protoplasts, (2) fusion of the protoplasts obtained from desired genotype, (3) culturing the hybrid cells, and (4) regeneration of hybrid plant. Mechanical or enzymatic methods can be used for the separation of protoplasts from plant tissue. However, the mechanical method is a laborious process that has some disadvantages such as low yield of protoplast and low protoplast viability. A plant cell consists of cell wall which has to be degraded if the protoplasts of the cell have to be manipulated as required. For this purpose, the plant cell is treated with enzymes, such as pectinase, macerozyme, cellulase, etc., that hydrolyze the plant cell wall. Since protoplasts are present in every plant cell, it can be theoretically isolated from all parts of the plant. But most successful isolations are made possible from the leaf of the plants.

Once purified protoplasts have been obtained from two different plant or tissue sources, various treatments can be given to induce them to fuse together. Generally, chemical agents or electrical manipulation is necessary to induce membrane instability that leads to protoplast fusion.

Polyethylene glycol (PEG) is used most frequently in conjunction with alkaline pH and high calcium concentrations. There are a number of steps in the fusion of plant protoplasts using PEG as a chemical facilitator. Another type of cell fusion that has emerged in recent years involves the manipulation of cell membranes by electrical currents. This process involves passing low-voltage electric pulses in a solution of protoplasts to be fused so that they line up for fusion. The protoplasts can be fused by subjecting it to brief exposure to high-voltage electric current which leads to alteration of membrane so that the adjacent protoplasts fuse. Electrofusion of plant protoplasts is often preferred over PEG fusion because it does not employ reagents that are toxic to the cells being fused. As with all other procedures, the conditions for electrofusion must be optimized for specific cell types to achieve maximum effectiveness. Typically, a yield of 20% or greater fusion products can be obtained by electrofusion of protoplasts compared to less than 1% fusion products with PEG [100].

Somatic cell fusion could overcome sexual incompatibility and long juvenility and may play a potential role in citrus genetic improvement, including producing directly or indirectly superior varieties, improving citrus scion and rootstock, or creating allopolyploids for triploid breeding [101, 102]. As Grosser and Gmitter [103] reported, this technique can facilitate conventional breeding, gene transfer, and cultivar development by bypassing some problems associated with the conventional sexual hybridization including sexual incompatibility, nucellar embryogenesis, and male or female sterility. Conversely, somatic hybridization is very promising for citrus rootstock breeding for combining genotypes having different tolerance to abiotic stress [104]. Citrus rootstock differs in terms of tolerance/resistance to abiotic stress

conditions. Incompatibility between some genotypes that have high level of tolerance to abiotic stress conditions limits the usage of traditional hybridization [105]. Oigawara et al. [106] reported the first intergeneric citrus hybrids obtained by combining embryonic callus of sweet orange and *Poncirus trifoliata* leaves via protoplast fusion. Grosser et al. [107] indicated that the regeneration of more than 300 plants obtained by protoplast fusion of Hamlin sweet orange and Flying Dragon trifoliata. The regenerated plants were determined as tetraploids. Kobayashi and Ohgawara [108] recovered tetraploid somatic hybrids by fusing the protoplast obtained from the embryonic callus of Trovita orange and leaf mesophyll protoplasts of Troyer citrange. Grosser et al. [109] reported tetraploid somatic hybrids obtained by fusing the protoplast via PEG method. They used several manipulations such as Cleopatra mandarin (*Citrus reshni*) + trifoliata orange (*Poncirus trifoliata* (L) Raf.), Acidless orange (*Citrus sinensis* (L) Osb.) + trifoliata orange (*Poncirus trifoliata* (L) Raf.), sour orange (*Citrus aurantium* L) + Flying Dragon trifoliata (*Poncirus trifoliata*), sour orange (*Citrus aurantium* L) + Rangpur lime (*Citrus limonia* Osb.), and Milam lemon + Sun Chu Sha mandarin (*Citrus reticulata* Blanco). Tetraploid plants were identified and propagated for further rootstock experiments. Ollitrault et al. [104] had reported the first intergeneric somatic hybrid obtained from protoplast fusion between *Citrus reticulata* + *Fortunella japonica* in France. The authors have regenerated approximately 100 plantlets by several manipulations (*C. reticulata* + *C. sinensis*, *C. reticulata* + *C. paradisi*, *C. reticulata* + *C. limon*, *C. reticulata* + *C. aurantifolia*, *C. reticulata* + *Poncirus trifoliata*, and *Citrus aurantium* + *Eremocitrus glauca*) and reported the possible use of these population as parental germplasm for both scion and rootstock breeding programs in citrus. Grosser et al. [99] indicated that the somatic hybrids obtained by *Citrus* + *Severinia* and *Citrus* + *Fortunella crassifolia* had lower performance as rootstocks, whereas promising performance was recorded from the scion grafted on somatic hybrids obtained from the manipulations of Acidless orange + *Atalantia ceylanica* and Nova mandarin + *Citropsis gillettiana*. Also, the researchers reported the dwarfing effects of somatic hybrids obtained by fusing the protoplast of sour orange + Flying Dragon and Cleopatra mandarin + Flying Dragon. Ollitrault et al. [16] had selected 11 allotetraploid somatic hybrids by using flow cytometry and molecular markers and propagated them for rootstock trials in order to investigate their tolerance to abiotic and biotic stress. Mourão Filho et al. [110] reported the root rot tolerance of the somatic hybrids ("Cleopatra" mandarin + "Volkamer" lemon, "Cleopatra" mandarin + sour orange, "Caipira" sweet orange + "Volkamer" lemon, and "Caipira" sweet orange + "Rangpur" lime). Somatic hybrid combinations involving sour orange or *Fortunella obovata* as one of the progenitors were intolerant to CTV. They suggested future field evaluations with somatic hybrids, especially those with tolerance to CTV.

In addition to these findings, tetraploid rootstocks usually have a built-in tree-size control mechanism due to some unknown physiological reaction with the diploid scion. Mourão Filho et al. [110] indicated that plants budded on tetraploid rootstocks are generally smaller, which could lead to reduced harvest costs and greater production efficiency. In Florida, more than 70 somatic hybrids that can potentially be used as rootstocks have already entered into commercial field trials. Preliminary results from these trials have shown that somatic hybrid rootstocks can produce adequate yields of high-quality sweet oranges (*Citrus sinensis* L. Osbeck) on small trees [111]. Ollitrault et al. [112] reported an intergeneric somatic hybrid



between Willow leaf mandarin and Pomeroy trifoliolate named as "Flhorag1." Dambier et al. [44] reported the agronomic evaluation of the Flhorag1 in Morocco. Valencia orange trees on Flhorag1 displayed the lowest growth followed by Carrizo citrange and Volkamer lemon in an agreement regarding tetraploid rootstocks controlling the tree size [109]. Flhorag1 also proved highly tolerant to iron deficiency (unpublished data of Çukurova University obtained within the framework of the INCO "CIBEWU" project).

Genetic transformation is also an attractive alternative technique for citrus genetic improvement. Almeida et al. reported that genetic transformation in Citrus has been obtained mainly from juvenile material such as embryogenic cells, epicotyl segments from *in vitro* germinated seedlings, and internodal segments from plants cultivated in the greenhouse due to a higher morphogenic ability compared to that of mature tissues [113, 114, 115, 116]. Peña et al. [18] concluded that the transformation efficiencies are generally low, and protocols are dependent on species, or even cultivar dependent. One of the limitations within this technology is low plant regeneration frequencies especially for many of the economically important citrus species [117, 118].

Another big area of biotechnology is DNA marker technology, derived from research in molecular genetics and genomics, which offers great promise for plant breeding. Owing to genetic linkage, DNA markers can be used to detect the presence of allelic variation in the genes underlying these traits. By using DNA markers to assist in plant breeding, efficiency and precision could be greatly increased. The use of DNA markers in plant breeding is called marker-assisted selection (MAS) and is a component of the new discipline of "molecular breeding" [119].

Genomic research in recent years led to the development of screening tools via marker-assisted selection, which enables much more efficient selection of superior recombinants improved for multiple traits from conventional breeding efforts. MAS can increase the efficiency of citrus breeding and may speed the release of new cultivars. In this section, the possibilities of using MAS method for early selection in citrus rootstock breeding programs will be discussed.

MAS can be very useful to efficiently select for traits that are difficult or expensive to measure, exhibit low heritability, and are expressed late in development. However, it is usually essential to confirm at certain points in the breeding process that the selected individuals or their progeny do in fact express the desired phenotype or trait. Marker types can be classified as morphological, biochemical, cytological, and DNA based (molecular). The successful application of MAS relies on the tight association between the marker and the major gene or quantitative trait locus (QTL) analysis responsible for the trait [120].

Carillo et al. [121] reported that many studies have focused on mapping QTLs for salt tolerance-related traits in rice because of its requirement for irrigation for maximum yield, its sensitivity to salinity, and its relatively small genome. Gmitter et al. [122] reported that a localized genetic linkage map of the region surrounding the citrus tristeza virus resistance gene was developed from *P. trifoliolate*. The authors indicated that the identification of markers tightly linked to CTV will enable citrus breeders to identify plants likely to be CTV resistant by indirect, marker-assisted selection, rather than by labor-intensive direct challenge with the pathogen. For early

selection in rootstock breeding program in citrus, Xu et al. [123] suggested that the feasible application of MAS in citrus rootstock breeding for citrus nematode resistance needs at least two genetic markers, each corresponding to related locus, in order to pyramiding the multi-genes associated or cofunctioned in controlling the citrus nematode resistance.

## 6. Conclusions

*Citrus* species are the most produced fruit crops and one of the most imported fruit groups subjected in both domestic and export markets. Citrus fruits can be produced in the tropical, semi-tropical, and subtropical climates with such a great market value. Factors limiting citrus growth in these climates significantly vary. Epidemic levels of disease and pest regarding the ecological conditions (temperature, relative humidity, and solar radiation) also remarkably differ. On the contrary, the existence of common abiotic stresses such as drought, salinity, and iron chlorosis in citrus-producing countries differs depending on the countries and the production areas of the countries. Rootstock is used for a successful citrus production against the abiotic stresses discussed.

In addition, searching for dwarfing rootstock, which enables high-density planting, has gained importance in terms of increasing the fruit yield per area, fruit quality, use of mechanical pruning, harvesting and decreasing pest, and disease management in citriculture. With this knowledge, we can say that there is no ideal rootstock which can combine all these traits. Hence, countries with different ecological conditions have to manage individual rootstock programs. In contrast, abiotic and biotic factors as well as the producer demands change depending on market conditions and new trends. Thus, breeding studies have to be persistent and managed by the current production situation and future demands.

In several breeding programs, existing rootstocks or genotypes that can be used as rootstocks in citrus production have been screened for abiotic stress conditions, and their current tolerance/resistance levels had been reported. These rootstocks currently have been used in citriculture depending on the ecological conditions of the producer countries. In addition, traditional breeding studies were carried out with these genotypes in order to combine their different tolerance/resistance characteristics in one genotype. Several successful rootstocks have been obtained so far by breeding programs. But traditional breeding takes at least 15 years from the begging of a cross-hybridization program until a new selected rootstock is released to the industry for a commercial use and limited by the complex reproductive biology of citrus. Therefore, rootstock breeders have begun to benefit using biotechnological methods in citrus breeding such as somatic hybridization, genetic transformation, and marker-assisted selection in recent years.

Genetic capacities of the parents or genotypes have great importance in breeding studies whichever method (traditional or biotechnological) is used, since the aim of breeding is to expose or combine the existing genetic capacity of parents. Therefore, screening both *ex situ* and *in situ* germplasm in the world regarding problems of citrus producer is necessary. Using promising genotypes obtained in screening studies carried out in germplasms in addition to



current citrus rootstock will result in an increase in breeding programs. As mentioned before, stress conditions are not stable and differ regarding the ecological conditions. Therefore, the sustainability of germplasm is necessary as long as human beings and citriculture exist.

Practical experience shows that abiotic stresses occur at high or low intensity in about all citricultural growing areas around the world. In this study, we have tried to explain the current situation of using citrus rootstocks for tolerance to abiotic stresses and breeding studies resulted and ongoing against abiotic stress in citrus production.

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# Cowpea Breeding for Drought Tolerance — From Brazil to World

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Additional information is available at the end of the chapter

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## Abstract

The aims of this chapter are to (i) present the importance of the cowpea crop, (ii) demonstrate problems related to drought, (iii) describe aspects related to flower structure and hybridization, and (iv) reveal how the genotype selection with tolerance to water deficit will promote increase of the yield in this culture. This chapter describes that Nigeria, Niger, and Brazil are the leading producers of cowpea crop worldwide, and this crop has a large influence on the economy of these countries. However, the drought problem can be frequently observed in areas with agricultural potential, with negative effects on production components and a consequent decrease of the yield in this culture. Breeding programs aimed at drought tolerance using selection strategies linked to genetic, biochemical, physiological, nutritional, and agronomic characteristics can help increase the yield and reduce losses promoted by the drought. In addition, flower structure and hybridization technique used in Núcleo de Pesquisa Vegetal Básica e Aplicada (NPVBA/UFRA) are presented, as well as populations are evaluated and plant management are explained in detail. This chapter describes the results obtained in other breeding programs aimed at drought tolerance and also explains the potential uses to increase the crop yield.

**Keywords:** *Vigna unguiculata*, production, drought, breeding

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## 1. Introduction

The cowpea [*Vigna unguiculata* (L.) Walp.] is cultivated widely in several countries due to its social, economic, and nutritional importance [1]. The aim of growing cowpea crop in such a huge quantity is to market its grains. These species are abundant in the regions of Latin

America, Africa, and Asia, while Nigeria, Niger, and Brazil are emerging as the leading producers of cowpea [2].

In Brazil, the production of cowpea is concentrated in the North and Northeast regions, because it presents interesting metabolic characteristics, such as low nutrient requirements, less water consumption, and well adapted to tropical environments, compared to other legumes [3-4]. Until the 1980s, the cowpea was almost exclusively grown by small and medium farmers. Currently, this culture seems to occupy other agricultural scenarios in areas with high irrigation potential, or/and also during the off-season; next only to the soybean crop, cowpea crops are being exploited by large producers by adopting new technologies [5-6].

The plants of various species under field conditions are constantly exposed to abiotic or biotic stresses, and suffer interactions that can significantly affect their performance [7], including the culture of cowpea [8]. Lawlor [9] points out that the main limiting abiotic factors of productivity in various plant species are drought, flooding, low and high temperatures, salinity, excess radiation, toxic heavy metals, and excessive macro- or micronutrients [10]. Drought is considered a major cause of the reduction of global agricultural production [11-12].

Drought is a frequently observed event and recurrent in areas with agricultural potential, mainly in tropical regions, causing an increase in temperature and decrease in the relative humidity [13]. This stress can be caused by irregularities in the distribution of rainfall [14-15] and/or inappropriate supplement irrigation for crops [16-17].

Thus, the water deficit acts as a limiting factor for both quality and production in several species with agronomic potential [18-20], including the cowpea [21]. The occurrence of drought during the growing and development and reproductive and maturity stages usually results in lower growth rates and development [22], flower abortion [23], and reduced grain production (GP) [24], thus complicating the reproduction process in the cowpea crop.

The decrease in the growth and development in cowpea plants observed during vegetative stages is related to negative interference periods of drought, which is caused by the lower weight of the aerial part of the plant [25], reduction in the expansion rate of leaf area [26], and severe reductions in gas exchange [27].

Drought induces molecular, physiological, biochemical, and morphological changes in cowpea plants [28-31], which are considered as adaptation strategies and survival mechanisms of the species [32]. Another problem normally connected to drought is the rise in temperature in the plant tissues due to reduced transpiration rate and consequent loss of effectiveness of thermoregulatory mechanism [33, 34]. This is due to water stress, as water is an essential element for the growth and development of all species of plants and plays an important role in photosynthesis, transport of organic solutes, and temperature control [35].

The increase in the rate of growth of the root system is a possibility to overcome the water stress in plants [36]; for, under such conditions, the stimulation of root growth in depth and the wetter areas of the soil profile [18] adaptations can check and control drought [26]. The root system of common bean often lies in surface when water availability is adequate [37]. Guimarães et al. [38] observed that the genotypes more tolerant to water stress had more

developed root systems compared with the susceptible genotypes. Additionally, Peña-Valdivia et al. [39], investigating common bean plants, describe negative changes related to the anatomy of the root, such as reductions in the xylem vessel and cross-sectional area of the root, in domesticated plants, while wild plants remained unchanged in structures and showed greater tolerance to drought.

The osmotic adjustment is another mechanism that can directly contribute to water retention in the plant tissue [40], for increasing the tolerance to dehydration [34]. This adjustment is accomplished by substances called osmoregulators such as carbohydrates and amino acids [41-42]. The accumulation of metabolites of plants provides a decrease in osmotic potential ( $\psi_s$ ) via a net increase in intracellular solute [43], which induces higher water retention in the tissue [44], and thus can control the plant cell elongation and expansion in growth regions [45].

The type of substances used in osmotic adjustment depends on the species and tissue [46]. However, the degree of adjustment depends on the speed at which the water deficit is applied, and osmotic adjustment is directly proportional to the intensity of water deficit [47]. The proline and glycine betaine are usually amino acids that act as osmotic adjusters in cowpea plants exposed to water deficit [48-49]. In water stress conditions, an increase in the synthesis of abscisic acid (ABA) in the roots is also observed, which is then transported to the shoot via the xylem [50].

In cowpea plants, overexpression of genes *CPRD 8*, *CPRD12*, *CPRD14*, *CPRD22*, and *CPRD46* conferring tolerance to water stress [28], as well as *VucAPX* genes, *VupAPX*, and *VutAPX*-connected *VusAPX* production of antioxidant enzymes [51], are reported, besides the high-level expression of *PvP5CS* gene associated with the production of proline, an amino acid that holds the osmotic adjusting function across species during drought.

The responses of cultivars and lines in cowpea are different in relation to water stress during the cycle [52]. In addition, there are factors such as the intensity and duration of drought that can intensify such stress [53-55].

The culture of cowpea requires basic and applied research related to the impact caused by water deficit, due to frequent and severe loss of production related to abiotic stress [56-58] and the high economic and nutritional importance to the producers and grain-consuming countries, such as Brazil [59-60].

The selection of genotypes of tolerance to drought has been carried out in several breeding programs [61-62]. However, the large number of genes involved in tolerance to drought [63], combined with the influence of the environment, hinders the selection of plants in segregating generations [53,57] and affects the evaluation lines/cultivars by virtue of the interaction, provided genotype environment often is significant [64].

The crosses between contrasting parenting have been widely used and allow to investigate the genetic control of quantitative traits such as drought tolerance [15,54]. Therefore, the characteristics described by Bastos et al. [8] are present in cultivars of cowpea, BRS Paraguaçu (drought tolerance) and Tracuateua-192 (sensitivity to drought), and are suitable for the purpose of this research and justify the hybridization between these cultivars, which are contrasting in relation to tolerance to drought.

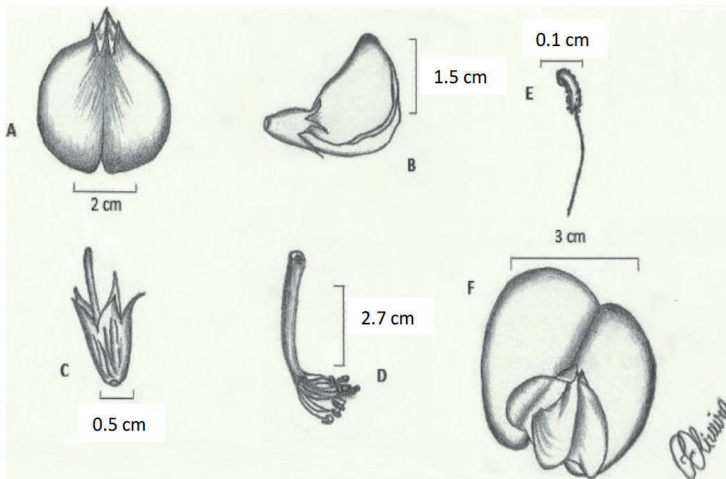
Adequate selection methods used in breeding programs of a species can reduce time and investment and maintenance of evaluating plants that could be previously discarded [65]. Thus, selection strategies linked to genetic, biochemical, physiological, nutritional, and agronomic characteristics can help due to the high detection efficiency and low cost [66]. In addition, the knowledge of the genetic control related to these characteristics will establish breeding strategies and selection of agronomically superior plants, with genes of tolerance to drought.

## 2. Objectives

The aims of this chapter are to (i) present the importance of the cowpea crop, (ii) demonstrate problems related to drought, (iii) describe aspects related to flower structure and hybridization, and (iv) reveal how the genotype selection with tolerance to water deficit will promote increase of the yield in this culture.

## 3. Flower structure in cowpea

In relation to flowers, the cowpea bean flower is hermaphrodite, deciduous, cyclic, dichlamydeous, and heterochlamydeous with zygomorphic symmetry (Figure 1). The five sepals and petals present are in free condition, denominated polysepalous and polypetalous, respectively [67].



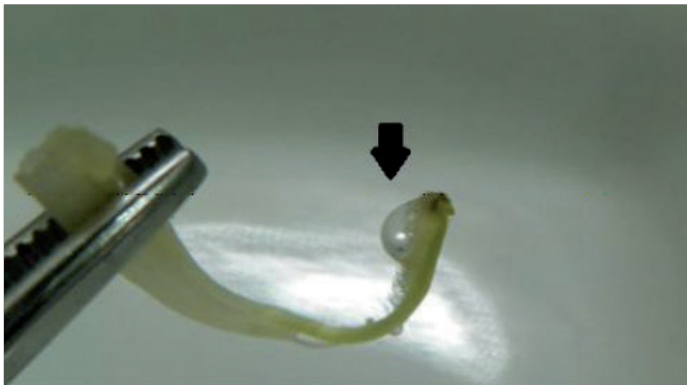
**Figure 1.** *V. unguiculata* floral pieces. (A) standard; (B) post-anthesis flower structure; (C) sepals; (D) stamen and pistil; (E) longer stamen; (F) keel formation [67].



Their corolla is papilionaceous; that is, it has an upper petal called standard, two side petals called wings and two lower, inner petals jointly called keel (Figure 1). One of the stamens is longer than the other nine (heterostemonous). Stamens are free, being diadelphous, with simple ramification; the anthers are free and basifixed, enclosed in relation to the corolla, longitudinally dehiscent and introrsed [67]. They are multicarpellary, syncarpous, with insertion in the terminal style. The hilum, chalaza, and micropyle are in the same straight line; that is, their ovule is classified as orthotropous [67].

#### 4. Aspects linked to artificial hybridization

The results described by Ribeiro et al. on aspects of the floral biology of *Vigna unguiculata* reveals that anthesis occurred between 05:00 and 05:30 h [67]. In pre-anthesis, at 4:00 h, the greenish flowers remained. In the stigma receptivity test, peroxidase action was observed at all times. As noted by Rocha et al., the floral opening of cowpea bean flowers begins around 05:30 h and continues until 09:30 h, when the stigma is still receptive (Figure 2) [68].



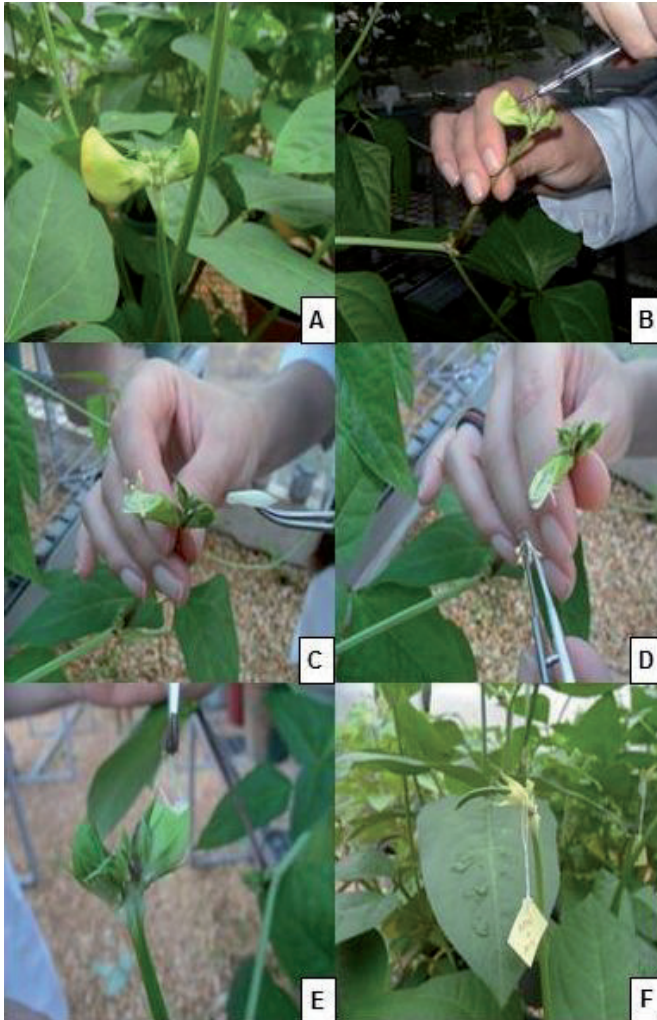
**Figure 2.** Peroxidase activity on *V. unguiculata* stigma, demonstrating receptiveness [67].

#### 5. Adaptations to hybridization in Brazilian conditions

The method of artificial hybridization described by Rachie et al. [69], as well as Zary and Miller Jr [70], was adjusted to conditions of infrastructure from Núcleo de Pesquisa Vegetal Básica e Aplicada (NPVBA) of the Universidade Federal Rural da Amazônia (UFRA) and climatic conditions of Northern Brazil (Figure 3), which used flower pollen collected in the morning (between 06:00 and 08:00 h) and stored in the refrigerator until use.

In the evening, the flower buds were emasculated and pollinated (16:30 and 17:30 h) (Figure 3). This method provided a higher percentage of successful pollination. This result is in

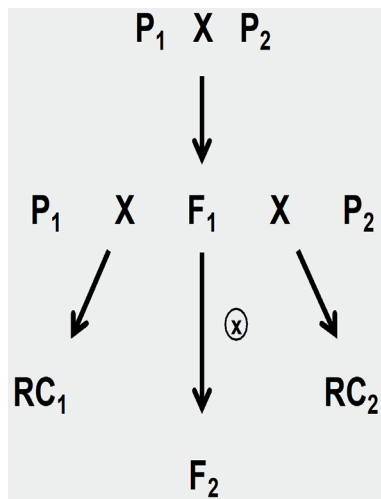
agreement with the Zary and Miller Jr [70] method, described by Teófilo et al. [71], who reported that the success of this method could be because the surface of the emasculated flowers are more receptive to pollen grains in the late afternoon, since, in this period, the temperature and moisture conditions are more appropriate.



**Figure 3.** Greenish flowers with flower from left in pre-anthesis stage (A); cut of standard in medium region (B); removal of the standard (C); removal of the anthers (D); application of pollen viable (E); successful pollination and pod formation (F).

## 6. Populations evaluated and plant management

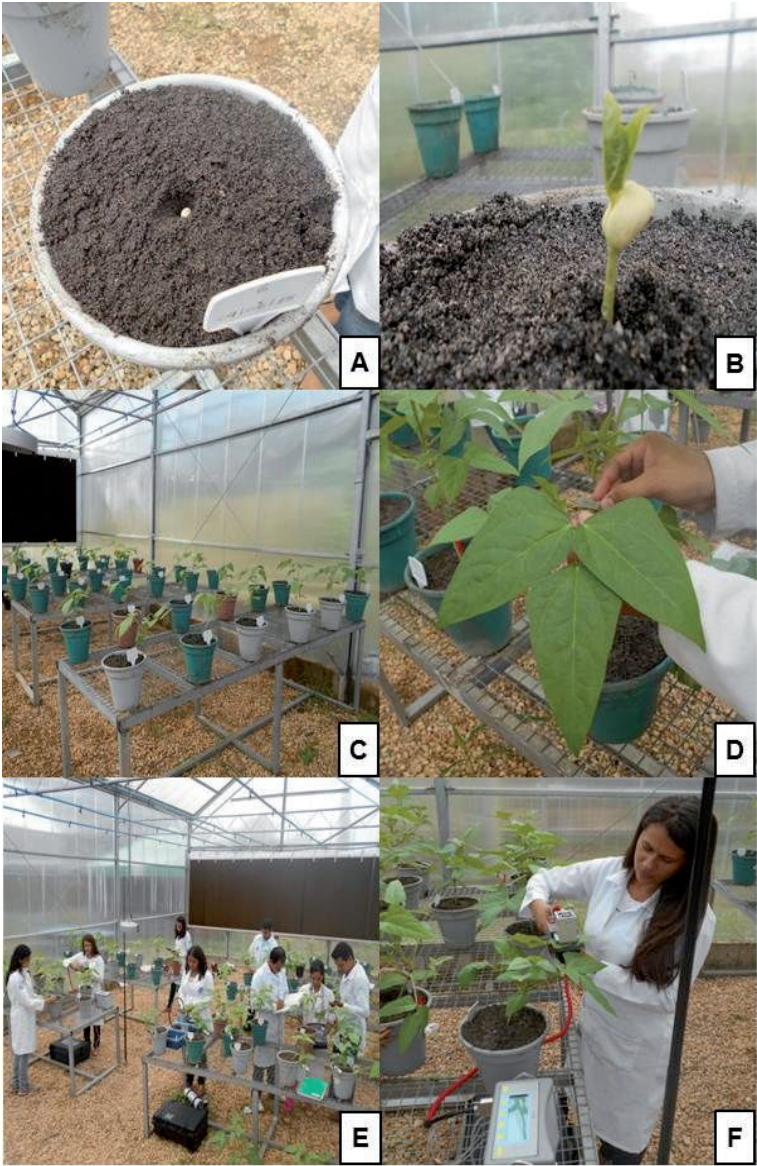
After hybridizations (Figure 4), the hybrid seeds were multiplied to obtain the  $F_2$ ,  $BC_1$ , and  $BC_2$  generations. In each pot, only one seed was placed. In this study, containers with capacity of 3 L containing holes in bottom were used, aiming to drain the excess water. The substrate was composed of a mixture of Plantmax® and sand in 3:1 ratio (v/v). Fertilization was performed according to exigencies of cowpea crop and previous substrate analysis, with the fertilization applied at regular intervals of 15 days until the 45th day after implementation experiment.



**Figure 4.** Scheme to obtain six populations ( $P_1$ ,  $P_2$ ,  $F_1$ ,  $F_2$ ,  $BC_1$ , and  $BC_2$ ) from crosses between LP 97-28 (low tolerance to drought)  $\times$  IPR-Uirapuru (high tolerance to drought).

## 7. Irrigation and water-deficit application

All plants were irrigated every day for 15 minutes at 10:00 and 13:00 h. The irrigation within the greenhouse was performed by a microsprinkler system, with a flow rate of  $10 \text{ L h}^{-1}$ . The three cycles of moderate stress were induced by irrigation suspension for four days at 25, 35, and 45 days after seedling emergence, whose periods coincided with the phenological stages  $V_3$ ,  $R_6$ , and  $R_8$ , respectively. In the cowpea crop, these periods described are related to vegetative, flowering, and pod filling stages, respectively (Figure 5).



**Figure 5.** Seed placed into substrate (A); *V. unguiculata* seedling (B); plants with 14 days (C); trifoliate leaf (D); data obtained during experiment (E); infra-red gas analyzer (F).

## 8. Results obtained in breeding programs aimed at drought tolerance

The study was conducted by Lobato et al. [72] with six populations, with two parents  $P_1$  and  $P_2$  and  $F_1$ ,  $F_2$ ,  $BC_1$ , and  $BC_2$  generations, derived from the cross between LP 97-28 (low tolerance to drought)  $\times$  IPR-Uirapuru (high tolerance to drought). Regarding production components, the grain production (GP) results reveal that the evaluated plants had values between 0.01 and 9.78 g. The low and high means for all populations were 2.30 and 6.86 g, respectively (Table 1). The  $F_2$  generation showed the best performance. The low and high values for variance were obtained in the parental  $P_1$  (1.51) and  $F_2$  (8.88) generations, respectively. In relation to the average weight of 100 seeds ( $W_{100s}$ ), the values ranged between 12.56 and 29.64 g. In addition, the low and high means were 18.49 and 21.83 g, with the greatest means observed in the  $F_1$  and  $P_2$  populations (Table 1). The greatest variance of 11.12 was obtained in the  $F_2$  generation. For the number of pods per plant (NPP), the plants studied had values between 1 and 19, and the lowest and highest means were 2.70 and 7.02, respectively (Table 1). The best result was found in the  $F_2$  generation, while the lowest value was observed in the  $BC_1$  generation. The highest variance of 8.08 was observed in the  $F_2$  generation (Table 1). For the number of seeds per pod (NSP), the plants collected in this study had values between 1.0 and 6.8. The low and high means were 4.17 and 4.64 in populations  $P_1$  and  $BC_2$ , respectively (Table 1). Additionally, the best result was found in generation  $BC_2$ . The low and high variances were 0.38 and 1.54 (Table 1) and were obtained in the  $P_2$  and  $F_2$  generations, respectively [72].

The estimated means of the segregating generations and the existence of high genetic variation ( $\sigma^2$ ) coupled with additivity indicated the presence of transgressive individuals. These findings enabled the selection of promising genotypes for drought tolerances higher than those of IPR-Uirapuru and LP 97-28, the parent lines in this study. In terms of the estimated variances in the study populations, the best performances were observed for the segregating generations ( $F_2$ ,  $BC_1$ , and  $BC_2$ ), which demonstrated higher values for all traits compared to the parent ( $P_1$  and  $P_2$ ) and  $F_1$  generations (Table 1). These results can be attributed to the large segregation of genes and, consequently, the higher amplitude in the distribution of the drought stress tolerance values, indicating genetic variation for the evaluated traits [73]. Similar results to those found in this study in terms of the GP of the  $F_2$  generation were reported by Szilagyi [74] for experiments with the common bean grown under adequate conditions (irrigation) and drought stress. This author studied production components in six populations derived from crosses between F332 and Ardeleana.

Higher variances for GP,  $W_{100s}$ , NPP, and NSP were observed in the  $F_2$  generation, revealing greater plant heterogeneity and suggesting great variability within this population. Genetic variability is extensively explored in breeding programs; it serves as the basis for selection and provides opportunities to establish a desired characteristic [75]. Smaller variances were obtained in the parents, confirming homozygosis in these populations due to the line and cultivar.



Regarding genetic control, the values of the phenotypic ( $\sigma_p^2$ ), environmental ( $\sigma_e^2$ ), genotypic ( $\sigma_g^2$ ), additive ( $\sigma_a^2$ ), and dominance variances ( $\sigma_d^2$ ) for grain production were 8.88, 2.97, 5.91, 5.75, and 0.16, respectively (Table 2). The genotypic variance corresponded to 66.6% of the phenotypic variance (total), and the additive variance accounted for 97.3% of the genetic variance. In W100s, the phenotypic, environmental, genotypic, additive, and dominance variances were 11.12, 2.55, 8.57, 7.66, and 0.91, respectively (Table 2). For this characteristic, the genotypic variance accounted for 77.1% of the total variance, while the additive variance corresponded to 89.4% of the existing genetic variance. In relation to NPP, the phenotypic, environmental, genotypic, additive, and dominance variances were 8.08, 2.33, 5.75, 4.73, and 1.02, respectively (Table 2). The phenotypic variance accounted for 71.2% of the genetic variance. Additionally, the additive variance corresponded to 82.3% of the genotypic variance. For the NSP, the phenotypic, environmental, genotypic, additive, and dominance variances were 1.54, 0.56, 0.98, 0.76, and 0.22, respectively (Table 2). The genotypic variance represented 63.7% of the phenotypic variance in this characteristic. The additive and dominance variances contributed to 77.6 and 22.4% of the genotypic variance, respectively [72].

The results indicate high contributions of additive variances in relation to genotypic variance and intense additive allelic interactions on all the evaluated traits. The existence of high additive variance suggests the identification of superior genotypes [76]. Typically, breeding methods that take advantage of high additive variance to obtain genetic gains are more important for the improvement of autogamous species, such as *Phaseolus vulgaris* [73].

According to this research, the use of additive variance is recommended as an indicator when studying GP, W100s, NPP, and NSP in the cross (LP 97-28  $\times$  IPR-Uirapuru), because it accounts for a significant portion of genotypic variance.

The estimates of broad-sense heritability ( $H^2$  %) ranged between 63.6 and 77.0% (Table 2), and the high and low values were found in the W100s and NSP characteristics, respectively. The estimates of narrow-sense heritability ( $h^2$  %) oscillated between 49.2 and 68.9% (Table 2), and the high and low values also corresponded to the W100s and NSP characteristics.

The average degree of dominance (add) values were 0.22, 0.48, 0.65, and 0.76 for the GP, W100s, NPP, and NSP characteristics, respectively (Table 2). The minimum number of genes (mng) that controlled the GP, W100s, NPP, and NSP characteristics were 4.7, 4.4, 8.6, and 5.5, respectively (Table 2).

The results related to broad- and narrow-sense heritabilities described in this study are high because studies involving populations are normally conducted under field conditions and high levels of environmental interference reduce genetic variances and produce lower heritabilities. Higher heritability coefficients may be caused by greater additive genetic variance, lower environmental variance, or minor interactions between genotype and environment [77]. Additionally, similar results for broad- and narrow-sense heritabilities indicate that the dominance effect is null. However, if the broad-sense heritability is higher than the narrow-sense heritability, the dominance effect is present [78].

LP 97-28×IPR-Uirapuru	GP(g)			W100s(g)		NPP		NSP	
	n	m	$\sigma^2$	m	$\sigma^2$	m	$\sigma^2$	m	$\sigma^2$
LP 97-28 (P <sub>1</sub> )	16	4.60	1.51	18.49	1.19	5.69	1.29	4.17	0.68
IPR-Uirapuru (P <sub>2</sub> )	18	4.48	1.89	21.83	1.37	4.39	2.13	4.60	0.38
F <sub>1</sub>	7	4.19	4.25	21.83	3.18	4.43	2.95	4.29	0.59
F <sub>2</sub>	166	6.86	8.88	21.29	11.12	7.02	8.08	4.16	1.54
BC <sub>1</sub>	10	2.30	4.92	19.29	6.44	2.70	5.34	4.54	1.43
BC <sub>2</sub>	39	3.31	7.07	20.52	8.13	3.44	6.09	4.64	0.88

**Table 1.** Number of evaluated plants (*n*), means (*m*), and variances ( $\sigma^2$ ) from grain production (GP), average weight of 100 seeds (W100s), number of pod per plant (NPP), and number of seeds per plant (NSP) obtained in six populations (P<sub>1</sub>, P<sub>2</sub>, F<sub>1</sub>, F<sub>2</sub>, BC<sub>1</sub>, and BC<sub>2</sub>), derived from cross between LP 97-28 × IPR-Uirapuru, Maringá-PR, Brazil, 2011 [72].

LP 97-28×IPR-Uirapuru	GP	W100s	NPP	NSP
Phenotypic variance ( $\sigma_p^2$ )	8.88	11.12	8.08	1.54
Environmental variance ( $\sigma_e^2$ )	2.97	2.55	2.33	0.56
Genotypic variance ( $\sigma_g^2$ )	5.91	8.57	5.75	0.98
Additive variance ( $\sigma_a^2$ )	5.75	7.66	4.73	0.76
Dominance variance ( $\sigma_d^2$ )	0.16	0.91	1.02	0.22
Broad sense heritability ( $H^2\%$ )	66.4	77.0	71.1	63.6
Narrow sense heritability ( $h^2\%$ )	64.7	68.9	58.5	49.2
Average degree of dominance ( <i>add</i> )	0.22	0.48	0.65	0.76
Minimum number of genes ( <i>mng</i> )	4.7	4.4	8.6	5.5

**Table 2.** Estimates of phenotypic variance ( $\sigma_p^2$ ), environmental variance ( $\sigma_e^2$ ), genotypic variance ( $\sigma_g^2$ ), additive variance ( $\sigma_a^2$ ), dominance variance ( $\sigma_d^2$ ), broad-sense heritability ( $H^2\%$ ), narrow-sense heritability ( $h^2\%$ ), average degree of dominance (*add*), and minimum number of genes (*mng*) related to grain production (GP), average weight of 100 seeds (W100s), number of pod per plant (NPP), and number of seeds per plant (NSP) obtained in six populations (P<sub>1</sub>, P<sub>2</sub>, F<sub>1</sub>, F<sub>2</sub>, BC<sub>1</sub>, and BC<sub>2</sub>), derived from cross between LP 97-28 × IPR-Uirapuru, Maringá-PR, Brazil, 2011 [72]. In differential of selection (DS), gain by selection (GS) and predicted genetic gain, the characteristics of grain production, average weight of 100 seeds, number of pods per plant, and number of seeds per pod had differential of selection (DS) values ranging from 1.49 to 4.85 (Table 3).

Characteristic	Genetic component					
	Mi	Ms	Ds	GS	GS(%)	PGG
GP	6.86	11.45	4.59	2.93	42.7	9.79
W100s	21.29	26.14	4.85	3.29	15.5	24.58
NPP	7.02	11.12	4.10	2.37	33.8	9.39
NSP	4.61	6.10	1.49	0.73	15.9	5.34

**Table 3.** Mean initial in F<sub>2</sub> generation (Mi), mean of selected plants in F<sub>2</sub> generation (Ms), differential of selection (Ds), gain by selection (GS), gain by selection expressed in percentage [(GS (%))], and predicted gain genetic (PGG) related to grain production (GP), average weight of 100 seeds (W100s), number of pod per plant (NPP), and number of seeds per plant (NSP) obtained in six populations (P<sub>1</sub>, P<sub>2</sub>, F<sub>1</sub>, F<sub>2</sub>, BC<sub>1</sub>, and BC<sub>2</sub>), derived from cross between LP 97-28 × IPR-Uirapuru, Maringá-PR, Brazil, 2011 [72].

The high and low values were obtained for the W100s and the NSP, respectively. In relation to gain by selection (GS), the GP, W100s, NPP, and NSP characteristics had values of 2.93, 3.29, 2.37, and 0.73, respectively (Table 3). When expressed as a percentage (% GS), the grain production trait had the highest value for gain by selection at 42.7%. The lowest value was found for the average weight of 100 seeds. The predicted genetic gain (PGG) values were 9.79, 24.58, 9.39, and 5.34 for the GP, W100s, NPP, and NSP characteristics, respectively (Table 3) [72].

Characteristic	GP	W100s	NPP	NSP
GP	-	0.36	0.96**	0.01
W100s		-	0.13	0.14
NPP			-	-0.20
NSP				-

Asterisks (\*\*) indicate significance to 0.01 of probability by the *t*-test.

**Table 4.** Coefficients of phenotypic correlation between grain production (GP), average weight of 100 seeds (W100s), number of pod per plant (NPP), and number of seeds per plant (NSP) obtained in six populations (P<sub>1</sub>, P<sub>2</sub>, F<sub>1</sub>, F<sub>2</sub>, BC<sub>1</sub>, and BC<sub>2</sub>), derived from cross between LP 97-28 × IPR-Uirapuru, Maringá-PR, Brazil, 2011 [72].

Regarding correlations between characteristics, results indicated that all characteristics were directly proportional (Table 4), except between the NSP and NPP, which were inversely proportional. Additionally, the results show a high correlation (0.96) between the NPP and GP. Moderate associations were found between the GP and W100s and the W100s and NSP within six generations (P<sub>1</sub>, P<sub>2</sub>, F<sub>1</sub>, F<sub>2</sub>, BC<sub>1</sub>, and BC<sub>2</sub>) derived from crosses between LP 97-28 and IPR-Uirapuru [72].

### 9. Final considerations

This chapter described that the leading producers of chickpea worldwide are Nigeria, Niger, and Brazil, and this crop has a large influence on economy of these countries. However, the drought represents a problem frequently observed in areas with agricultural potential, with negative repercussion on production components and consequent decrease of the yield in this culture. Breeding programs aiming tolerance to drought using selection strategies linked to genetic, biochemical, physiological, nutritional, and agronomic characteristics can help increase the yield and reduce losses promoted by the drought. In addition, flower structure and hybridization technique used in Núcleo de Pesquisa Vegetal Básica e Aplicada (NPVBA/UFRA) were presented, as well as populations evaluated and plant management were explained in detail. This chapter described the results obtained in other breeding programs aimed at drought tolerance and also explained the potential uses to increase the crop yield.



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# Sunflower Breeding for Resistance to Abiotic and Biotic Stresses

Dragan Škorić

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/62159>

## Abstract

Due to a specific structure of its main organs (root, stem, leaves, and head), sunflower can be successfully grown on marginal soils and in semiarid conditions, and it is more resistant to abiotic stresses, than other field crops. Unfortunately, it is very sensitive to biotic stresses.

In sunflower breeding for resistance to abiotic stresses, the greatest progress has been made in selection for drought resistance. Breeders use over 30 different parameters in sunflower screening for drought resistance, with physiological ones being the predominant type. The best breeding results have been achieved using the phenomenon of stay-green, with the added bonus that this method incorporates into the cultivated sunflower not only drought resistance but resistance to *Macrophomina* and *Phomopsis* as well. The diversity of the wild *Helianthus* species offers great possibilities for increasing the genetic resistance of the cultivated sunflower toward abiotic stresses. In using wild sunflower species in sunflower breeding for drought resistance and resistance to salinity, best results have so far been achieved with *H. argophyllus* and *H. paradoxus*, respectively. In addition to the use of wild *Helianthus* species, sunflower breeding for abiotic stress resistance should also make more use of molecular breeding techniques. More progress has been made in sunflower breeding for heat resistance than in that for cold resistance. Specific breeding programs dealing with sunflower resistance to mineral deficiency and mineral toxicity have yet to be established.

Concerning biotic stresses, the main problem in sunflower cultivation is caused by fungal diseases. Genetic variability of cultivated sunflower is very low and deficient in disease-resistance genes. Due to wild sunflower species of the *Helianthus* genus, genes that confer resistance to certain diseases were discovered and incorporated into the genotypes of the cultivated sunflower. Based on the wild species, genes were found that confer resistance to *Plasmopara halstedii*, *Puccinia helianthi*, *Verticillium dahliae*, *V. albo-atrum*, and *Erysiphe cichoracearum*. Furthermore, wild sunflower species provide a high level of tolerance (field resistance) to *Phomopsis/Diaporthe helianthi*, *Macrophomina phaseolina*, *Albugo eragropognis*, and *Alternaria* ssp. Sources of resistance to other harmful diseases are sought after within wild sunflower species.

With the use of one wild species of *H. annuus* from Kansas (USA.), genes conferring resistance to a group of imidazolinone (IMI) or sulfonylurea herbicides were discovered. Moreover, similar genes were found through induced mutations. These sources of resistance provide successful control over a broad spectrum of weeds, which infest sunflower crops, including broomrape.

The growth of the parasitic weed sunflower broomrape (*Orobancha cumana* Wallr) is a major issue in sunflower production, especially in Central and Eastern Europe, as well as in Spain. Six races of broomrape have been detected (A, B, C, D, E, and F) and dominant resistance genes ( $Or_1$ ,  $Or_2$ ,  $Or_3$ ,  $Or_4$ ,  $Or_5$ , and  $Or_6$ ) were found in wild sunflower species. During the last 4–10 years, new virulent races of broomrape emerged in several European countries. Geneticists and breeders work on finding the sources of resistance to the new broomrape races in wild sunflower species.

Numerous insect species cause economic damages during sunflower production, especially in North America (the homeland of sunflower). *Homoeosoma* species are the most widespread insects that infest sunflower. *Homoeosoma nebulella* infests sunflower in Europe and Asia, while infestation with *H. electellum* poses a major problem in USA, Canada, and Mexico. Based on the use of wild sunflower species *H. tuberosus*, genes conferring resistance to *Homoeosoma* species were incorporated. Sunflower has an armored layer in the hull, which provides resistance to this insect. Sources of resistance to other economically harmful insects are sought after.

New methods in biotechnology, particularly marker genes, have been frequently used in breeding for abiotic and biotic stresses.

**Keywords:** Abiotic and biotic stresses, breeding, interspecies hybridization, resistance, sunflower, wild species

## 1. Introduction

### 1.1. Sunflower breeding for resistance to abiotic stresses

Abiotic stresses not only determine the geographical and regional distribution of crops but also dictate if a potentially arable piece of land can actually be used for cultivation. According to an estimate, 24.2% of the world's geographic area is potentially arable. However, only 10.6% of the geographic area is under actual cultivation, while the rest is not available for cultivation due to one or more abiotic stresses [1]. According to the same author, drought is the main abiotic factor, as it affects 26% of the arable area. Mineral toxicities/deficiencies are second in importance, while frost stands third. Drought is the most limiting of all abiotic stresses, and it affects well over one-third of the soils worldwide. Plants that manage to survive the effects of drought stress show a decrease in fertility, yield, and product quality [2].

Characterization of drought tolerance is very complex and interrelated to many factors. Drought is a multidimensional stress affecting plants at various levels of their organization. Sunflower is grown in a number of countries on so-called marginal soils, often in semiarid conditions where almost every year an abiotic stress of one kind or another is present acting

as a limiting factor on crop production. However, of all field crops, sunflower is best able to withstand drought conditions, primarily on account of the structure of its organs [3].

Drought is the main cause not only of differences between mean yield and potential yield but also of yield variations from year to year and therefore of yield instability [2].

Using the results of our own studies and those of other authors, the present chapter discusses the progress that has so far been made in sunflower breeding for resistance to abiotic stresses and indicates possible future directions in this area of sunflower research.

## 1.2. Sunflower breeding for resistance to drought

Previous experiences in sunflower cultivation have shown that drought can be a limiting factor in realizing the potential of a variety or a hybrid.

In sunflower breeding for resistance to drought, just like in the other crops, a number of physical and morphological parameters are at play. The accumulation of genes for these parameters in a single genotype makes it possible to increase resistance to drought [4].

Škorić [5] states that sunflowers must be resistant to both soil and air drought, that is, to high temperatures during flowering (pollination) and the oil synthesis stage. The ways to achieve this desired goal are as follows: a more efficient root system, a certain systemic composition of the main organs, and resistance to certain diseases (*Macrophomina phaseoli*). In addition to efficient water use, the root system must have the ability for efficient nutrient use under stress conditions.

On the one hand, resistance depends on the selection of genotypes whose flowering and maturity end before the occurrence of stress (early maturity).

On the other hand, mechanism of drought resistance incorporates the modification of certain physiological and morphological parameters, which enables a more efficient use of water reserves during the period of stress. The mechanism manifests itself through a more aggressive root system or water use reduction via a more efficient stomatal apparatus plus the interaction of these factors.

The inheritance of tolerance of drought based on high osmotic pressure was found to be controlled by partial dominance and overdominance. The inheritance of drought tolerance measured by temperature shock was found to be based on nonallelic interaction of genes contained in the system of partial dominance [6].

Soil drought limits water uptake and consumption by plants. Transpiration intensity decreases strongly, which, in combination with high air temperature, leads to overheating of plants. The protective reaction of plants against water shortage is the increased ability of cells to retain water. Respiration intensity typically increases under the influence of drought. Prolonged drought forces the plants to reduce the energy efficiency of respiration [22].

Fulda *et al.* [8] used their own results and those of other authors to conclude as follows. Obviously, water stress acclimation is a multigene acclimation, in which many different physiological processes and many drought stress-inducible genes are involved. Functionally,

these gene products can be distinguished into osmolyte synthesis, protection factors for macromolecules (chaperons, LEA/dehydrin type genes), proteases, membrane proteins (aquaporins, transporters, detoxification enzymes (glutathione-S-transferase (GST) and superoxide dismutase (SOD)), and genes of regulatory proteins such as transcription factors (TFs), protein kinases, and protein phosphatases. Although the alterations in all of these processes related to drought stress have been widely investigated in many model species and a few crop species, reports on sunflower are limited.

Studying the influence of water deficit and canopy senescence pattern on sunflower root functionality during the grain-filling phase, Lisanti *et al.* [9] have concluded that both water deficit and intrinsic canopy senescence dynamics can profoundly affect root functionality during grain-filling. The effects of these factors and their interactions, especially under drought, on yield merit focused attention in future research.

According to Singh [1], drought seems rather difficult to define and more difficult to quantify. For example, the common criteria used in the various definitions are precipitation, air temperature, relative humidity, evaporation from free water surface, transpiration, wind, air flow, soil moisture, and plant conditions. A working definition of drought may be "the inadequacy of water availability, including precipitation and soil moisture storage capacity, in quantity and distribution during the life cycle of a crop to restrict the expression of its full genetic yield potential".

Therefore, under conditions of drought, water stress develops in the plants as the demand exceeds water supply; this may occur due to atmospheric or soil conditions and is reflected in a gradient of water potentials developed in the soil/soil-root interface and the leaf, the transpiring organ. Thus, moisture stress may be defined as the inability of plants to meet the evapotranspirational demand. Moisture stress is likely to develop to a different rate in different plant organs along this gradient [10].

Drought resistance may be defined as mechanism(s) causing minimal loss of yield in a drought environment relative to the maximum yield in a constraint-free, that is, optimal environment for the crop. However, it does not exist as a unique heritable plant attribute. The various mechanisms by which a crop can minimize yield loss due to drought are grouped into the following three categories:

1. drought escape
2. dehydration avoidance, and
3. dehydration tolerance [1]

Drought escape describes the situation where an otherwise drought-susceptible variety performs well in a drought environment simply by avoiding the period of drought. Early maturity is an important vehicle for drought escape, suitable for environments subjected to late-season drought stress [1].

Early sunflower hybrids generally have lower leaf area index (LAI), lower total evapotranspiration, and lower yield potential than the later ones. According to Škorić [11], early sunflower

hybrids are most often susceptible to *Macrophomina*, and thus in cases where there is an early occurrence of drought such hybrids may become affected, thus nullifying any positive effect early maturity may bring.

Dehydration avoidance is the ability of a plant "to retain a relatively higher level of hydration under conditions of soil or atmospheric water stress." Therefore, the various physiological, biochemical, and metabolic processes involved in plant growth and yield production are not internally exposed to stress, but they are protected from water stress [10]. The common measure of dehydration avoidance is the tissue water status as expressed by water or turgor potential under conditions of water stress. This can be achieved by either reducing transpiration (such plants are often called water savers) or increasing water uptake (such plants are often termed as water spenders). Wild species are readily classifiable as water savers and water spenders, but crop plants ordinarily exhibit a combination of both features, probably as a result of selection by man.

Drought not only reduces the rate of photosynthesis but also directs the photosynthetic metabolism toward increased formation of low-molecular weight compounds such as alanine, hexoses, and malic acid [12]. When the drought ends, sunflower plants are capable of again having a high rate of photosynthesis, thus compensating for the negative effects of water deficiency.

As sunflower plants respond to drought, the free proline content of their leaves increases, because proline, due to its structure, increases the water retention capacity of the cell [13].

When breeding for dehydration avoidance, it is highly important that a considerable attention is paid to parameters such as reduced transpiration, osmotic adjustment, abscisic acid (ABA), cuticular wax, and leaf characteristics (leaf pubescence, altering the leaf angle, and leaf rolling). It is also especially important to find ways to increase water uptake by creating a more powerful, deeper, and well-branched root system [14].

#### 1.2.1. Sources of drought resistance

Several types of germplasms are used in sunflower breeding for drought resistance:

1. landraces;
2. cultivated hybrids and varieties;
3. wild species of the genus *Helianthus*; [15]; and
4. genetically engineered germplasm.

Use of landraces and cultivated hybrids and varieties has produced some positive results, but not to the extent that would secure stable sunflower production under drought conditions. The best results in increasing the drought resistance of cultivated sunflower have been achieved using wild species of the genus *Helianthus*.

Over the last 10–14 years, highly drought-tolerant germplasms based on *H. argophyllus*, which have a commercial value, have been created in various breeding centers.

Research and characterization of physiological mechanisms in wild sunflower are just beginning. Škorić [16] suggests that in breeding for drought tolerance, there should be a greater effort to expand the use of other wild species such as *H. deserticola*, *H. hirsutus*, *H. maximiliani*, *H. Tuberosus*, and others.

#### 1.2.2. Using different traits in sunflower breeding for drought resistance

Škorić [7] reported that over 30 different parameters were used in the study of drought resistance and breeding for drought resistance in sunflower. Among these, the most frequently used were physiological parameters.

Chimenti *et al.* [17] reported that high osmotic families extracted more water from the profile during the stress period and had greater grain yield and leaf area duration than families with a low degree of osmotic adjustment. The same authors concluded that osmotic adjustment can contribute to post-anthesis drought tolerance in sunflower through increased water uptake, reduced impact on grain number, grain size, and greater leaf area duration.

Andrei [18] concluded that high self-fertility (24–49%) in some hybrids ensured a greater stability in sunflower yield under stress conditions.

Studying the influence of drought stress on growth, protein expression, and osmolyte accumulation in sunflower, Fulda *et al.* [8] reported that osmolyte analysis revealed an accumulation of glucose (24–30-fold), inositol (20–30-fold), proline (10–20-fold), fructose (3–6-fold), and sucrose (4–4-fold) in extracts from leaves of drought-stressed plants. Changes in protein expression of drought-stressed versus control plants were detected in colloidal Coomassie-stained 2D-polyacrylamide gel electrophoresis (PAGE).

Sato *et al.* [19] studied the correlation between the responses of leaf expansion and hypocotyl elongation to water deficit in sunflower genotypes. Based on the results obtained, they reported that the response of hypocotyl growth to water deficit ranged between 31 and 48%, while that of leaf growth ranged between 40 and 63%. There was a significant positive correlation ( $p < 0.01$   $R^2 = 0.61$ ) between both responses. The correlation was also significant using Pearson's correlation test ( $p < 0.04$ ,  $r = 0.78$ ).

Petcu *et al.* [20] studied physiological traits for the quantification of drought tolerance in sunflower and determined as follows. The reduction in leaf area, shoot size, and biomass accumulation of sunflower seedlings under water stress conditions determined the increase in root/shoot ratio. This suggests that for young plants the main sink was survival. In a late stage of vegetation, the root/shoot ratio decreased under drought stress in some hybrids but increased in others, suggesting that for mature plants the main sink was the yield. The physiology work has focused on morpho-physiological traits induced by drought and associated with drought tolerance of plants and the elaboration of screening methods for rapidly measuring drought tolerance using plants in an early stage of vegetation.

Based on the results of Škorić [7, 11], practical results in sunflower breeding for drought resistance have been achieved by using the stay-green phenomenon. Here, we should warn

that in the selection of lines on the basis of stay-green criteria, only lines with a high degree of self-fertility should be looked for, otherwise a wrong choice of genotypes will be made.

The use of the stay-green criterion involves the selection of not only genotypes resistant to drought but also those resistant to *Macrophomina*, which tends to be a problem under stress conditions. Also, genotypes resistant to *Phomopsis* may be simultaneously selected, as confirmed by the inbred lines Ha-48, Ha-22, CMS-1-40, PH-BC-2-91, PR-ST-3, RHA-SES, RHA-483, etc. as well as the hybrids made from these lines, which combine several resistance systems. Vrănceanu [21] confirmed the validity of using the stay-green criterion in the selection for drought resistance [22].

Petrović *et al.* [23] concluded that nitrate reductase activity and free-proline accumulation rate, which underwent large modifications in plants under water stress, may serve as parameters for the evaluation of sunflower genotypes for drought tolerance.

Working on the determination of water stress index in sunflower, Orta *et al.* [24] found statistically significant correlations between CWSI (crop water stress index) calculated from single leaf temperatures on the one hand and stomatal resistance, leaf area index, and available water in the root on the other.

Early sunflower hybrids generally have lower leaf area index, total evapotranspiration, and yield potential than the later hybrids. However, according to Škorić [11], early hybrids are typically sensitive to *Macrophomina*, so in the case of an early manifestation of drought they become infected and thus the advantage of earliness is nullified.

Some breeders believe that drought avoidance can be achieved by developing very early sunflower hybrids or by moving the sowing date (early or late sowing) in order to avoid the dry period. Dehydration avoidance can be achieved in several ways, for example, by selecting genotypes with reduced transpiration (water savers) or by increasing the uptake of available water from the soil by a powerful root system (water spenders).

Characteristics that appear to be correlated with drought tolerance include deeper rooting depth and more efficient root uptake of water, tolerance to high osmotic pressure, low transpiration rates, and plant ability to recover after wilting under heat stress.

The genetics of sunflower resistance to drought has not been studied sufficiently, despite numerous attempts and use of different plant characteristics. It appears safe to say that the drought resistance (tolerance) is controlled by a set of genes.

### 1.3. Sunflower breeding for resistance to salinity

Abiotic stress can be generated by mineral salts, which affect a considerable portion of the global arable land. Salinity ranks second after moisture stress. This stress may occur in the form of a specific mineral deficiency or toxicity, or as accumulation of an excess amount of soluble salts in the root zone [1].

Sunflowers are grown on low-to-medium-saline soils in many countries. These countries face soil salinity as a serious limiting factor in sunflower production. However, it should be

remembered that there are several wild *Helianthus* species that naturally grow on saline soils. These species are important sources of genes for resistance to salinity. Breeders should apply effective screening methods in order to identify the wild species that possess genes useful in breeding for salinity resistance and equally effective breeding methods to transfer these genes into cultivated sunflower genotypes [22].

Seiler [25] stated that several wild species of *Helianthus* are native to salt-impacted habitats and may possess genes for salt tolerance. The same author reports that Chandler and Jan [26] evaluated three wild *Helianthus* species for salt tolerance, namely *H. paradoxus*, *H. Debilis*, and *H. annuus* population native to salty desert areas, and obtained the following results. *Helianthus debilis* tolerated a salt concentration about the same as cultivated sunflower, wilting at an NaCl concentration of 240–400 mM. The wild ecotype of *H. annuus* had a higher tolerance, with some plants surviving the NaCl concentration of 800 mM. *Helianthus paradoxus* was highly salt tolerant, with some plants surviving at 1300 mM of NaCl. Salt tolerance was a dominant trait in hybrids between *H. paradoxus* and cultivated *H. annuus*, which did as well as the wild parent.

The emergence percentage, emergence index, shoot length, and shoot fresh weight can be used as selection criteria for salt tolerance in sunflower at the seedling stage [27].

Tolerance of sunflower genotypes to salinity has been investigated by a number of researchers. Prakash *et al.* [28] found that turgor is not correlated with salt tolerance. The accumulation of proline shows a higher impact on tolerance to salinity. Since callus development, seed germination, and vigor are associated, the former could be a more reliable index of salt tolerance.

The involvement of turgor and proline in salt tolerance seems to be doubtful [29]. Prakash *et al.* [28] stated that turgor cannot be related to salt tolerance. However, proline accumulation seems to be more due to the effect of salinity.

Evidently, using *H. paradoxus* and possibly some other wild *Helianthus* species, sunflower breeders can successfully achieve high resistance to salinity. It is important to determine the selection criteria that can be applied in the breeding program, and these can be cell survival, seed germination, dry matter accumulation, leaf death or senescence, leaf ion content, leaf necrosis, root growth, osmoregulation, etc. [1].

#### 1.4. Sunflower breeding for resistance to mineral deficiency and mineral toxicity

Sunflowers require only 10 macroelements (C, O, H, N, P, K, S, Ca, Fe, and Mg) and 6 microelements (B, Mn, Cu, Zn, Mo, and Co) for their growth and development. Air and water are the sources of carbon, oxygen, and hydrogen. The rest of the elements are taken up from the soil or fertilizers and are divided into primary elements, secondary elements, and microelements [14]. Sunflower nutrition has been the subject of many books and scientific papers, which have established optimum levels of each individual macro- and microelement needed for the normal growth and development of sunflower on different types of soil. There is also voluminous literature on the deficiencies or excess levels (toxicity) of individual elements and how they affect sunflower growth and development.



Studying the diversity of elements in sunflower inbred lines, Sarić *et al.* [30] came to the conclusion that the genetic specificity for mineral nutrition is manifested not only through different contents of mineral elements but also through their distribution into individual plant organs.

As there are unfortunately no major breeding programs anywhere in the world that deal specifically with sunflower resistance to mineral deficiency and mineral toxicity, sunflower breeders should consider a possibility of establishing one or more such programs. They would have to choose appropriate breeding methods and targets, define selection criteria, and select potential resistance sources (most likely wild *Helianthus* species) [16].

### 1.5. Sunflower breeding for heat resistance

Singh [1] made a very good definition of the heat and cold resistance, which reads: "Each plant species, more particularly genotype, has an *optimum range of temperatures* for its normal growth and development: the specific temperatures would depend not only on the genotype but also on the stage of growth and development of a given genotype. When temperature moves beyond this optimal range, it generates *temperature stress*, i.e., temperature interferes with the performance. Temperature stress may be grouped into the following three categories: (1) heat stress, (2) chilling stress and (3) freezing stress."

Sunflower is characterized by high adaptability to high temperatures. At high temperatures, sunflower intensifies the process of transpiration so that its leaves remain relatively cool. Transpiration rate can be increased only if sufficient water is supplied and this calls for a deep and well-developed root system. Therefore, the choice of genotypes with a deep and powerful root system is an important criterion in the selection for sunflower tolerance to high temperatures [22].

Another important criterion is the tolerance to intensive transpiration. For the environments in which high air temperatures frequently occur at the flowering stage, breeders should select genotypes capable of producing large quantities of pollen and maintain pollen viability under such conditions. It is also important for the pistil and its stigma, or for the disk flowers on the whole, to be tolerant to high temperatures, which ensures pollination and seed formation [22].

Yet another criterion for the selection of genotypes adapted to climates with high temperatures and air and soil drought is the capacity for high seed (formation) filling rate and rapid synthesis of oil in response to stress conditions.

In order for sunflower breeders to be able to determine the right breeding methods, targets, and selection criteria and to choose their breeding materials for selection for heat resistance, they must have a detailed knowledge of how sunflower organs respond to high temperatures. Sunflower is exposed to high temperatures in arid and semiarid conditions, which have been prevalent in much of Europe in 2007. High temperatures may be accompanied by high, but also low humidity levels.

The present knowledge on sunflower heat resistance allows sunflower breeders to define their selection criteria more easily and to search for sources of heat resistance in wild *Helianthus* species.

Breeding for resistance to high temperatures should be combined with selection for drought resistance. Intensive breeding programs on sunflower heat resistance should be organized in countries where excessive temperatures are a regular occurrence. Selection for heat resistance is an integral part of many breeding programs and is often combined with breeding for increased productivity and resistance to dominant diseases and drought [16].

### 1.6. Sunflower breeding for resistance to low temperatures (cold)

In many environments, crop productivity is limited by low temperatures. When temperatures remain above the freezing level, that is,  $>0^{\circ}\text{C}$ , it is called chilling, while freezing describes temperatures below this level, that is,  $<0^{\circ}\text{C}$ .

For sunflower, it is important to increase its resistance to cold in the early stages of growth and development, that is, at germination, emergence, and the stage of two to three leaf pairs, so as to enable successful early sowing. Cold resistance at maturation should be increased as well in order to enable sunflower growing at higher altitudes and in colder regions. Sources of cold resistance should be sought exclusively in the wild *Helianthus* species that are found growing wild in the mountains where winters are harsh and springs are cold [16].

Apart from wild *Helianthus* species, induced mutations can also be successfully used as sources of resistance to low temperatures.

Excellent results in the development of sunflower genotypes resistant to cold were achieved by Kalaydzhyan *et al.* [31, 32], who applied induced mutations by chemical mutagens, first of all DMS. Resistance to low temperatures was tested in 44,000 seeds of about 2,000 mutagenic progenies by planting them in late fall/early winter. Some 499 plants from 72 mutagenic progenies (0.91%) survived the harsh winter and low temperatures (down to  $-20^{\circ}\text{C}$ ). The following mutants showed highest resistance to low temperatures:

- in the case of M-1248 (progenies of 40–43), the overwintering rate was 63%;
- in the case of M-1976 (progenies of 14–20), the overwintering rate was 48%;
- in the case of M-2002 (progenies of 44–64), the overwintering rate was 42%;
- in the case of the cultivar Radnik (control), the freezing rate (death) was 100%.
- These mutants should be subjected to the cold test in the climatic chamber in order to obtain more reliable results.

In any case, Kalaydzhyan *et al.* [31, 32] evidently developed a unique germplasm, which can be used for the development of winter genotypes and genotypes tolerant to low temperatures. Unfortunately, sunflower geneticists and breeders around the world seem to be unaware of these outstanding results.

## 1.7. Sunflower breeding for tolerance to herbicides

In the past decade or so, significant results were achieved in sunflower breeding for resistance (tolerance) to herbicides from the class of imidazolinones and some herbicides from the class of sulfonylureas (SU).

Acetolactate synthase (ALS), also called acetohydroxyacid synthase (AHAS), is the first enzyme in the biosynthesis of three vital amino acids in plants: valine, leucine, and isoleucine. Four different classes of herbicides inhibit ALS, thus causing the herbicidal effect. The most common are imidazolinones and sulfonylureas. They have been widely used since their introduction in the early 1980s, and now they constitute one of the major weed control mode-of-action classes for many crops. Resistant (tolerant) plants rapidly metabolize the herbicide in herbicidally inactive form. Sensitivity is likewise due to the lack of metabolic detoxification (Stoenescu, personal communication).

Advantages of ALS-inhibiting herbicides are as follows: very low application rate, broad spectrum of weed control (broad leaf and grassy weed species), broad range of crop, selectivity, etc.

### 1.7.1. Development of IMI-resistant sunflower hybrids

A wild population of annual *H. annuus* from a soybean field in Kansas that had been repeatedly treated with imazethapyr for 7 consecutive years developed resistance to the imidazolinone and sulfonylurea herbicides [33]. Resistance to imazethapyr and imazamox herbicides has great potential for producers in all regions of the world for controlling several broad-leaved weeds.

Miller and Al-Khatib [34] reported that the USDA-ARS (NDSU) research team quickly transferred this genetic resistance into cultivated sunflowers and released public "IMISUN" lines in 1998. At the same time, Alonso *et al.* [35], IFVC research team, Novi Sad, and several private companies in Argentina incorporated IMI resistance from the wild population of *H. annuus* L. from Kansas into their elite lines and developed the first IMI-resistant hybrids [22]. Genetic stocks IMISUN-1 (oil maintainer), IMISUN-2 (oil restorer), and IMISUN-3 (confection maintainer) have been developed and released [36]. Miller and Al-Khatib [34] also released one oilseed maintainer and two fertility restorer breeding lines with imidazolinone herbicide resistance.

Malidža *et al.* [37] reported having transferred resistance to imidazolinones from the wild *H. annuus* L. from Kansas into the elite line HA-26 using three generations per year (one in the field and two in the greenhouse). They stated that the resistance was controlled by a single partially dominant gene. Alonso *et al.* [35] were among the first in the world to transfer genes from the wild *H. annuus* L. population collected in Kansas into a cultivated sunflower genotypes resistant to the herbicide imazethapyr, which also 100% controlled (destroyed) broomrape in sunflowers.

Studying the mode of inheritance of resistance to imidazolinone herbicides by using F<sub>2</sub> and test-cross population, Bruniard and Miller [38] concluded that the resistance was controlled

by two genes, a major gene having a semidominant type of gene action (*Imr1*) and a second gene (*Imr2*) with a modifier effect when the major gene is present.

Resistance in sunflower can only be achieved with homozygosity (*Imr1 Imr1*, *Imr2 Imr2*) of both resistance genes in inbred line or in a hybrid [38].

Sala *et al.* [39] reported having obtained a new source of IMI resistance, CLHA-PLUS, developed by means of induced mutations. The line was obtained through ethyl methanesulfonate mutagenesis and selection for the herbicide imazapyr. Also, the authors proved at the molecular level that CLHA-PLUS is different from *Imr1* and that both of them are allelic variants of the locus AHASL1 [40].

It has been shown experimentally that the gene CHLA-PLUS has a higher degree of IMI resistance than the gene *Imr 1 Imr 2*. Breeding centers wishing to use the CHLA-PLUS gene for breeding purposes have to sign a contract on its use with the company BASF. At the same time, BASF provides a protocol for screening for resistance at the molecular level (CLEARFIELD® Protocol SF30).

The recently established CLEARFIELD® (a BASF trademark) Production System for Sunflower provides growers with a new technology, which ensures broad-spectrum postemergence grass and broad-leaved weed control combined with high-performing sunflower hybrids from leading seed companies or public institutions.

BASF Corp. has also established two testing systems which serve to approve IMI-resistant sunflower hybrids as CLEARFIELD®, based mainly on relative tolerance compared with a standard resistant hybrid: Global and Country Qualification System.

Over the last 5 years, there has been a rapid spread of IMI (CLEARFIELD®)-resistant hybrids in the USA, Argentina, and especially central and eastern Europe, where new races of broomrape, which can be successfully controlled by this technology, have emerged.

### 1.7.2. Development of hybrids resistant to sulfonylurea (tribenuron-methyl)

Simultaneously with sunflower breeding for IMI resistance, work has been started on the development of hybrids resistant to herbicides from the tribenuron-methyl group of sulfonylureas. To date, two resistance sources have been discovered:

The first one was derived from SU-resistant wild *Helianthus annuus* plants collected from the same area in Kansas where IMI resistance was found. The USDA-ARS (NDSU) research group incorporated this genetic resistance into cultivated sunflower and released public lines SURES in 2001 [41].

At the same time, sunflower breeders in various breeding centers (public and private) in the world introduced the sulfonylurea resistance gene into their elite lines, and thus created resistant hybrids.

The second SU resistance was detected by DuPont within an artificial mutagenesis project conducted in the early 1990s. This material was reselected, purified, and tested by Pioneer/

DuPont during 1998–2000. Several mutation events were evaluated and selectivity to the sunflower mutation event SU7 was confirmed for a narrow range of SU herbicides.

Also, in SU-resistant hybrids, it is necessary that both parent lines possess resistance, because of the partial domination in inheritance of this trait.

#### 1.7.3. *The use of molecular techniques in sunflower breeding for resistance to abiotic stress*

Molecular studies as part of sunflower breeding for resistance to abiotic stress should be focused on the recognition of chromosomal segments carrying genes that contribute to the determination of tolerance, provide the possibility to partition the character, and can be used as a tool for an efficient manipulation of the breeding material. For this purpose, genetic maps of neutral molecular markers, such as isozyme and restriction fragment length polymorphism loci, can be an efficient tool for the determination of useful genes [42].

Belhassen *et al.* [43] and Cellier *et al.* [44] were among the first to use molecular techniques in sunflower breeding for resistance to abiotic stress.

Belhassen *et al.* [43] started breeding for drought tolerance from an interspecific cross with *H. argophyllus*. Four cycles of divergent selection using the physiological criterion of leaf cuticular transpiration (relative water loss) allowed the production of two contrasting genotypes: T– (low level of leaf cuticular transpiration) and T+ (high level of leaf cuticular transpiration). Field experiments showed better yield tolerance index combined with good potential yield for T– hybrids in some locations. Physiological analyses conducted in the field and in controlled conditions allowed to distinguish the two genotypes for only one parameter – osmotic adjustment. Molecular comparison revealed the existence of a cDNA differentiating T– from T+. This cDNA has high homology with an amino acid transporter. A quantification of the amino acid concentrations during water deficit in T– and T+ lines showed that the T– plants accumulate significantly more proline than T+ ones. Using this cDNA, RFLP and STS analysis allowed the differentiation of the two lines.

Cellier *et al.* [44] studied a sunflower genotype showing drought tolerance in field conditions (R1 genotype) and another exhibiting drought sensitivity (S1 genotype). They found that R1 tolerance was characterized by a delay of both wilting and decrease of leaf water potential. To analyze R1 tolerance at a molecular level, they isolated different cDNAs (named SDI for Sunflower Drought Induced) corresponding to transcripts accumulated in water-stressed R1 leaves by subtractive hybridization. The analysis of transcript accumulation in both genotypes upon drought stress suggested a differential expression in the *sdi* genes. Absciscic acid-mediated induction in the tolerant genotype was observed for four of the *sdi* genes and was found to differ among them. Sequence analysis of SDI clones showed high identity with known proteins, including nonspecific lipid transfer proteins (nsLTPs), early light-inducible proteins (ELIPs), or dehydrin, predicted to be involved in various physiological processes.

Arce *et al.* [45] studied sunflower atypical transcription factors and miRNAs playing a key role in responses to abiotic stresses. In order to achieve the desired results, they used a series of molecular biology techniques. These techniques and strategies include database analysis, phylogenetic tree construction, screening of genomic DNA libraries, isolation of cDNA clones,

expression studies using northern blots, western blots, and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), functional analyses using plant transformation, both stable and transient, confocal microscopy, and microarrays.

Among their findings was the conclusion that transcription factors are proteins able to recognize and bind specific DNA sequences present in the regulatory regions of their target genes. Upon binding, entire signalization cascades are induced or repressed and the plant can adapt itself, at least temporarily, to the adverse conditions to which it is subjected.

Based on the copious results, Arce *et al.* [45] made the following conclusions.

The most amazing results obtained during these studies and other current studies are related to the divergence in structure and function of TFs and miRNAs found in sunflower, apparently conserved in some cases in other *Asteraceae* species but not in model plants. The release of the genomic sequence together with the advance in transformation techniques will certainly help to better understand how sunflower evolved to be adapted to abiotic stress factors and which novel regulating molecules are playing key roles in such an adaptation.

Alberdi *et al.* [46] studied the relationship between a set of molecular markers (amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR)) and leaf expansion parameters under water-deficit conditions in a cross of two public sunflower lines of contrasting response, in its  $F_2$  and  $F_{2.3}$  progenies, and in an independent  $F_8$  recombinant inbred line (RIL) population.

Based on phenotypic trials (two in growth chambers –  $F_3$  and  $F_{2.3}$ ) and experiments in a greenhouse (RIL population), certain leaves collected during these experiments were used for DNA extraction. Using a set of 60 SSR and 41 AFLP markers, they achieved significant results, which may be useful for the development of molecular markers for assisted selection in breeding programs oriented to generate new cultivars with improved adaptation to water stress conditions.

Liu and Jan [47] closely studied the results of molecular studies about abiotic stresses in light of their own as well as other authors' research. They concluded that approaches using molecular biology, functional genomics, transcriptome, and proteomics have been used to identify genes or quantitative trait loci (QTLs) and proteins correlated with the network of the response to such stresses, which will provide knowledge for the development of hybrids with resistance or tolerance to them. Some wild species grow in locally extreme environments providing an opportunity to study species from these habitats.

Studying the phenomenon of salt tolerance in sunflower, Lexer *et al.* [48] identified an EST that codes for the Ca-dependent protein kinase with maps to a salt-tolerance QTL in sunflower.

### 1.8. Conclusions

Due to the basic structure of its main organs (root, stem, and leaves), sunflower is more resistant to abiotic stresses than other field crops. Therefore, it is usually grown on soils of lower quality ("marginal soils") and in semiarid and arid conditions, where it is often exposed to abiotic stresses.

When it comes to sunflower breeding for resistance to abiotic stresses, the greatest progress has been made in selection for drought resistance. The progress was achieved by using various criteria and parameters, but the most headway was made by using physiological parameters.

The best and the most affordable method for testing sunflower for drought resistance is the use of “stay-green” character. By using “stay-green” in sunflower selection for drought resistance, the selection for *Macrophomina* and *Phomopsis* resistance is made at the same time.

Wild sunflower species of *Helianthus* are successfully used in selection for drought resistance. *Helianthus argophyllus* is most commonly used in selection for drought resistance via interspecies hybridization. Thus, new germplasms have been developed in a number of breeding centers. Moreover, several more wild species deserve to be used in selection for drought resistance. The use of molecular breeding techniques enables faster and more efficient achievement of desired results in sunflower resistance to drought.

Significant results in sunflower selection for salinity resistance have been obtained by the use of *H. paradoxus* via interspecies hybridization.

Cold resistance can be increased by using certain wild species of sunflower, but especially induced mutations.

Wild species of sunflower are insufficiently used in selection for high temperature resistance, that is, heat resistance, as well as mineral deficiency and mineral toxicity resistance.

By using a population of wild *H. annuus* L. and induced mutations, great headway in sunflower selection for resistance to herbicides from the imidazolinones and sulfonylureas (tribenuron-methyl) group has been made. Sunflower resistance to broomrape (*Orobancha* spp.) has also been achieved.

## 2. Sunflower breeding for resistance to biotic stresses

Concerning biotic stresses in sunflowers, it can be safely concluded that diseases caused by different fungi present the most serious problem. Broomrape, the parasitic angiosperm, is in the second place, viruses and bacteria in third and fourth [22].

### 2.1. Sunflower diseases

The original variability of the cultivated sunflower is very narrow and different in genes applicable in selection for the improvement of different agronomic traits, especially those conferring resistance to diseases.

Diseases are a limiting factor in the production of sunflower in all continents where it is grown. Different diseases are dominant in different growing regions, depending on the prevailing environmental conditions. Some diseases cause economic damage to sunflower in all sunflower-growing regions of the world. More than 30 different pathogens that attack sunflowers and cause economic loss in production have been identified so far (Table 1). Sunflower breeders

have achieved significant results in finding genes for resistance or high tolerance to certain diseases in wild species and incorporating them into cultivated sunflower genotypes possessing high combining ability [22].

Disease	Pathogen
Downy mildew	<i>Plasmopara halstedii</i>
Broomrape	<i>Orobanche cumana</i>
White rot	<i>Sclerotinia sclerotiorum</i>
Stem canker	<i>Diaporthe helianthi</i>
Alternaria blight	<i>Alternaria helianthi</i> , <i>A. helianthificiens</i>
Rust	<i>Puccinia helianthi</i>
Phoma black stem	<i>Phoma macdonaldii</i>
Virus	<i>Sunflower chlorotic mottle virus</i>
Verticillium wilt	<i>Verticillium dahliae</i>
Charcoal rot	<i>Macrophomina phaseolina</i>
White blister rust	<i>Albugo tragopogonis</i>
Fusarium wilt	<i>Fusarium</i> spp.
Rhizopus head rot	<i>Rhizopus</i> spp.

**Table 1.** The most common sunflower diseases

Wild sunflower species have been a valuable source of resistance genes for many of the common pathogens of the cultivated sunflower. The relative severity of individual diseases varies widely, depending on climate and host cultivars. Breeding for resistance often is the most effective means of control. Sources of resistance or improved levels of tolerance for most diseases are available among the cultivated sunflower and the wild species of *Helianthus* [49].

Changes in the racial composition of certain pathogens have also been caused by the introduction of hybrids in commercial production, which are substantially more homogeneous with respect to the previous period when genetically heterogeneous open-pollinating varieties were grown.

Vear [50] recommended for efficient disease control in future breeding programs to combine vertical and horizontal resistance if available. If not, marker-assisted selection should be used to combine QTLs with different additive defense mechanisms [22].

Galina Pustovoit [51] evaluated new cultivars based on interspecific hybridization (*H. tuberosus* × cultivated sunflower) – Progress, October, Yubileyniy 60, and Novinka. Based on the results achieved in the field and by inoculation, the author concluded that the new cultivars possess group immunity, that is, resistance to downy mildew, rust, *Macrophomina*, *Phoma*, and broomrape.



To be successful in breeding for disease resistance, the sunflower breeder must be thoroughly acquainted with general principles of resistance breeding, major approaches to management of resistance genes, stability of sunflower resistance to certain pathogens, monitoring of interactions between the host (sunflower), pathogen and the environment, and resistance types (vertical and horizontal). Finally, he has to have an adequate germplasm at his disposal, select a method of breeding, and develop a strategy for achieving the desired goal [22].

The aim of this research is to review biotic stresses in sunflower, indicate their significance, and reveal the sources of resistance and methods of selection in order to achieve the desired goal.

#### 2.1.1.1. Downy mildew [*Plasmopara halstedii* (Farl.) Berl. et de Toni])

Downy mildew [*Plasmopara halstedii* (Farl.) Berl. et de Toni]) occurs in all regions around the world in which sunflower is grown as a major oil crop. Downy mildew occurs with light intensity in years with a wet spring.

Downy mildew control was successfully maintained with dominant genes for a long period. This period roughly corresponds to the presence of only two races of downy mildew, the European race, controlled by the dominant gene *Pl<sub>1</sub>*, and North American, controlled by the *Pl<sub>2</sub>* gene. Unfortunately, changes took place in the past 14 years and there occurred a number of new races. These new races of downy mildew were registered in France, Hungary, USA, Argentina, and several other countries [22].

Viranyi [52] reported that the most detailed and up-to-date list of global distribution of *P. halstedii* pathogens has been compiled by Gulya [54] in a paper presented at the 2nd International Downy Mildew Symposium, Kostelec, Czech Republic. In the accurate overview, he comprised as many as 34 pathotypes (races), an unbelievably high number considering the fact that in most sunflower-producing countries from just a few to 12 well-distinguished virulence phenotypes exist. Europe, France, Germany, and Spain reported the highest numbers but the pathogen is rather diverse in the USA, Canada, and in South Africa as well. Furthermore, there are five *P. halstedii* pathotypes (300, 330, 710, 730, and 770) that are universally distributed globally, recorded from North and South America, Europe and Africa. Apart from the quantitative aspect of virulence, it is interesting to consider the dynamics of diversity as well, that is, the changes in a given region over time. In this respect, France leads with the highest number of new pathotypes arisen in the last 6–7 years [53].

Here, it should be mentioned that genes for resistance to the new races were quickly found in wild species and promptly transferred into genotypes of the cultivated sunflower [55]. An international set of differential lines has been made which makes it possible to determine which downy mildew races are present in a certain region. The set of differentials is supplemented with new lines as new downy mildew races occur [50].

The dynamism of changes in downy mildew races may be illustrated by the fact that, conclusive, with 2011, at least 18 downy mildew races have been determined in the world (100, 300, 304, 307, 314, 330, 700, 703, 704, 710, 711, 714, 717, 721, 730, 731, 770,...).

The testing of breeding materials by inoculation methods is in constant improvement and continuous progress. These issues have been dealt with by a large number of researchers, including Gulya *et al.* [56], Gulya *et al.* [57], Jouffret *et al.* [58], Tourvieille de Labrouhe *et al.* [59], Molinero-Demilly *et al.* [60], and others.

Tourvieille de Labrouhe *et al.* [61] reported that, in addition to major genes, nonrace-specific resistance contributes to the expression of resistance to downy mildew as well. The study also showed that the nonrace-specific resistance is inherited independently of major genes. Furthermore, Vear *et al.* [62] concluded that the inheritance of nonrace-specific resistance is under additive control. The authors reported that two QTLs may explain 42% variation in field reaction to downy mildew. This form of resistance was mapped as belonging to linkage groups 8 and 10. At the same time, they argued that this quantitative resistance is not related to any of the known major resistance gene clusters.

Also, Vear *et al.* [63] have developed a procedure for the development of new B-lines and parallel conversion into the *cms* form from source population. We should also mention here the procedure (scheme) of Vear *et al.* [64] for introgressing *Pl* genes into elite B-lines by backcrossing and simultaneous conversion into the *cms* form while performing resistance screening at the molecular level. This method significantly shortens the cycle of *Pl* gene introgression into elite lines.

According to Tourvieille de Labrouhe *et al.* [65] and Vear *et al.* [62], breeders should develop a strategy of simultaneous selection for nonspecific resistance and major gene resistance along with requisite use of molecular markers.

According to Seiler [49], complete resistance to the downy mildew pathogen was found in annual species *H. annuus*, *H. argophyllus*, *H. debilis*, and *H. petiolaris* and perennial *H. decapetalus*, *H. divaricatus*, *H. eggertii*, *H. giganteus*, *H. xlaetiflorus*, *H. mollis*, *H. nuttallii*, *H. scaberrimus*, *H. pauciflorus*, *H. salicifolius*, and *H. tuberosus* [66].

Diploid perennial species *H. divaricatus*, *H. giganteus*, *H. glaucophyllus*, *H. grosseserratus*, *H. mollis*, *H. nuttallii*, and *H. smithii* and their interspecific hybrids were resistant to downy mildew [67].

With the rapid improvement of molecular techniques and their use in plant pathology, new developments have opened new insights into research on fungal biology, detection technology, and genetics and host–pathogen interactions. For example, Hammer *et al.* [68] in Germany, using different approaches, were successful in detecting fungal structure from sunflower host tissues.

### 2.1.2. White rot [*Sclerotinia sclerotiorum* (Lib.) de Bary]

White rot is a major problem in countries with a humid climate or in years with an extremely wet summer. The fungus itself is polyphagous. It attacks over 360 plant species, which increases its variability and makes the selection for resistance difficult [69]. The major problem in the selection are the three types of the diseases (on the root, stem, and head) controlled by different mechanisms of resistance [11].

Sunflower stalk and head rot incident by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered the most important disease of the crop in many parts of the world. Since cultural practices or fungicides are insufficient to control the disease, efforts are being made by breeders to develop resistant or tolerant cultivars. This may explain the dominance of publications dealing with various aspects of resistance [52].

When breeding sunflower for resistance to all three forms of *Sclerotinia* attack, it is necessary to combine two or three different tests [70].

Mancel and Shein [71] found that *Sclerotinia* isolates taken from different plant species differed in the degree of virulence. They also found that sunflower isolates that had been repeatedly subcultured in the laboratory were significantly less virulent than isolates recently obtained from sunflower.

When we discuss the three types of sunflower infection by *Sclerotinia*, it is easy to achieve high tolerance to the mid-stalk infection by selecting genotypes resistant to lice [11]. Young leaves of such genotypes are not injured by lice and therefore these plants avoid infections.

Using four different tests for the evaluation of *Sclerotinia* resistance (basal stem infection, ascospore, and oxalic acid injection into the back face of the head), Baldini *et al.* [72] found that the inbred line 28R was most tolerant to the basal stem and white head rot infections and it also showed the best performance in oxalate and culture filtrate tests, which indicated the presence of a specific resistance to oxalate.

Van Becelaere and Miller [73] tried different inoculation procedures for evaluation of resistance to *Sclerotinia* head rot. According to their results, the best method involved the spraying of heads at the beginning of flowering with 4 cm<sup>3</sup> of a suspension of ascospores, which contained 4000 ascospores per milliliter, and covering the heads with brown paper bags immediately after inoculation. Measurements of inoculation could begin as early as 34 days after the inoculation.

Vear *et al.* [50] studied the virulence of 10 *Sclerotinia* isolates. They found differences in in vitro growth rate and sclerotia production as well as some highly significant isolate and genotype effects. They concluded that the available resistance in sunflower genotypes has partial, nonrace-specific, and horizontal characteristics and that it should be durable.

Using an in vitro screening test based on callus induction to evaluate *Sclerotinia* resistance, Drumeva *et al.* [74] found that the test allowed the identification of the breeding material with high to moderate resistance to the pathogen.

When developing inbred lines, sunflower breeders should take note of the results of Van Becelaere [75], who found that the general combining ability (GCA) effects of female lines were relatively larger than the GCA effects of male lines, which indicated that, at least in that particular research, the female lines had a greater influence on the resistance of the hybrids.

When considering the methods of selection for white rot tolerance, recurrent selection and pedigree method were found to produce the best results.

Vear *et al.* [63] used the pedigree method to select sunflower heads resistant to *Sclerotinia*. They applied the ascospore test on F<sub>2</sub> and F<sub>4</sub> plants and the mycelium test on F<sub>3</sub> plants. Their results showed that in all cases there was a variation in the level of resistance among F<sub>3</sub> families. The gains in relation to their parents ranged from 24 to 61%.

Vear *et al.* [76] applied 14 cycles of recurrent selection to a sunflower restorer population developed in 1978 and they obtained significant results. The mycelium test was used in the first three cycles and a combined test with a suspension of ascospores in the subsequent cycles. About 80% reduction of the infected area was achieved in the fourth cycle. In the 12th cycle, the latency index (a measure of incubation period) in the ascospore test was doubled. Simple regression provided the best relation with this cycle, indicating that further increase in the degree of tolerance was possible.

Christov [66] and Christov *et al.* [77] reported that higher-ploidy perennial species (hexaploid and tetraploid species) exhibited greater susceptibility than the diploids, with *H. glaucophyllus*, *H. divaricatus*, *H. salicifolius*, and *H. mollis* having the highest frequency of healthy plants. Tolerance to *Sclerotinia* was observed in the perennials *H. eggertii*, *H. pauciflorus*, and *H. smithii* and annuals *H. annuus*, *H. argophyllus*, *H. petiolaris*, and *H. praecox* [78].

Interspecific hybrids based on *H. nuttallii*, *H. giganteus*, and *H. maximiliani* were reported to show resistance against stem infection by Henn *et al.* [79]. Miller and Gulya [80] developed four maintainer and four restorer oilseed lines with improved tolerance to *Sclerotinia* stalk rot. The inbred line HA 410 released by Miller and Gulya [80] derived from a wild perennial hexaploid, *H. pauciflorus* (= *rigidus*), had a moderate tolerance to stalk rot. *Sclerotinia* root rot tolerance was observed in perennials *H. mollis*, *H. nuttallii*, *H. resinosus*, and *H. tuberosus* [81].

Among the perennial species, resistance to *Sclerotinia* was observed in population of *H. tuberosus*, *H. divaricatus*, *H. hirsutus*, *H. maximiliani*, *H. mollis*, *H. nuttallii*, *H. occidentalis*, and *H. rigidus* (= *pauciflorus*) grown under natural infection conditions [82].

*Sclerotinia* head rot tolerance was observed in perennials *H. resinosus*, *H. tuberosus*, *H. decapetalus*, *H. grosseserratus*, *H. nuttallii*, and *H. pauciflorus* [83–85].

In the past decade, advances were made in the research of *Sclerotinia* resistance at the molecular level, particularly in the marker-assisted selection [86, 50, 62, and many others]. The new methods are expected to provide significant help to sunflower breeders [86].

### 2.1.3. Sunflower rust (*Puccinia helianthi* Schw.)

Rust is the second most important sunflower disease considering its global distribution. The disease causes economic loargophyllussess in sunflower production in North and South America, Australia and Africa. Based on our own observations, rust is present in several countries in Asia (China, India, Iran, Kazakhstan, and others), but its racial composition has not been determined yet. Fortunately for Europe, the local rust population is fairly stable. Rust races were studied most extensively in North America. Sackston [87] determined four North American races, 1, 2, 3, and 4. Race 4 was identified by Yang [88] and race 6 by Lambrides and Miller [89].

Antonelli [90] and Senetinner *et al.* [91] studied sunflower resistance to an Argentinean rust isolate, clone 340, and found that the lines MP 447, MP 444, and LC 74/74-20620 were resistant to it and that the resistance was controlled by a single dominant gene.

Hugues *et al.* [92] studied the occurrence and distribution of rust in Argentina in the period 1982–2008. Their results indicated that resistant cultivars were stable in terms of rust resistance. They also concluded that a single rust pathotype existed in central and southern sunflower-growing regions of Argentina, which was in contrast to previous studies.

In Africa, the determination of rust races in sunflower was done only in Mozambique. Using differential lines from Canada and USA, Vicente and Zazzerini [93] found that the rust race 4 was present in Mozambique.

In Europe, rust has been studied on a limited scale. Most of the work had been done at VNIIMK, Krasnodar. Studying various methods of inoculation by rust, Galina Pustovoi and Slyusar [94] concluded that growing a mixture of resistant genotypes in spatial isolation completed by selection of resistant plants was the most appropriate method.

Miller *et al.* [95] tested 343 genotypes for resistance to rust and found that 12 genotypes were resistant to race 4. The authors also found that the lines HA-R1, HA-R3, HA-R4, HA-R34, and 647-1 shared the same locus,  $R_4$ , while the line HA-R2 had a different one that was named  $R_4$ .

Kochman and Goulter [96] proposed a system for identification of rust races in sunflower, and examined the slow-rusting phenomenon and resistance gene pyramiding to control sunflower rust.

Sendall *et al.* [97] studied the diversity of *Puccinia helianthi* pathosystem in sunflower in Australia at the molecular level and found a set of 24 lines and determined putative resistance genes.

Regarding the methods of artificial inoculation, Gulya and Maširević [98] provided a detailed description of inoculation techniques for evaluating sunflower resistance to rust under laboratory conditions (greenhouse experiments) as well as under field conditions. They also ranked the differential lines in three sets: set one (S-37-388, CM90RR, and MC29), set two (P-386, HA-R1, and HA-R2), and set three (R3-HA, HA-R4, and R4-HA).

Wild species of the genus *Helianthus* are a rich gene pool for further identification of resistance genes and their use to forestall the emergence of new races of *Puccinia helianthi*.

#### 2.1.1.4. Stem canker (*Phomopsis*) *Diaporthe helianthi*

In the past three decades, *Phomopsis* has become the most destructive disease on the global scale. Its large-scale occurrence was first registered in the Vojvodina Province (Serbia) and Romania in 1980, when it caused large economic damage to sunflower production. Soon afterwards, it was registered in most sunflower-growing countries in Europe (France, Hungary, Slovakia, Bulgaria, Ukraine, Russia, and Italy). In the early 1980s, its presence was reported in the USA, Canada, Argentina, Uruguay, Australia, Iran, and some other countries [22].

The first significant results in sunflower breeding for resistance to *Phomopsis* were achieved in Serbia and Romania.

Škorić [99] reported that of 4000 inbred lines and 2000 experimental hybrids, only four lines exhibited field resistance to *Phomopsis*. Two of these lines had been derived from interspecific hybrids (cultivated sunflower  $\times$  *H. tuberosus*): one was obtained from a cross of *H. argophyllus*  $\times$  Armavirski 9344 and the restorer line SNRF-69 was derived from a local population from Hungary.

Based on extensive research, Vrânceanu *et al.* [100] found that the sunflower resistance to *Phomopsis* is of the horizontal type and that it is positively correlated with the stay-green phenomenon. The authors reported that, of all Romanian hybrids, Select has the highest degree of tolerance to *Phomopsis*.

Škorić [99] found that three female lines (Ha-22, Ha-74, and Ha-BCPL) and the restorer line SNRF-6 are field resistant to *Phomopsis*. Resistance was transferred to the hybrids NS-H-43, NS-H-44, and H-NS-44 developed from these lines. The same author also reported that *Phomopsis* resistance is positively correlated with *Macrophomina* and *Phoma* resistance as well as with drought tolerance.

Vrânceanu *et al.* [101] concluded that partial dominance is expressed in the inheritance of *Phomopsis* resistance in some cases, while additive inheritance is much more frequent. The same authors found that the stay-green stem at the ripening stage is positively correlated with *Phomopsis* resistance.

Much work has been done lately on the use of molecular markers in breeding for *Phomopsis* resistance.

Studying recombinant inbred lines derived by crossing LR4-17 (resistant) with HA89 (susceptible) at the molecular level, Langer *et al.* [102, 103] concluded that unlinked segments carried major QTLs for different components of resistance, and that the resistances of leaves and stems could be pyramided with a marker-assisted selection.

Molecular studies on the intraspecific diversity of this fungus using intergenic spacer sequence analysis revealed a high homology among French/Yugoslavian and among Italian isolates [104]. The phylogenetic tree obtained from the aligned data revealed three separate groups. The analysis also showed that all isolates originating from countries with regular and severe outbreaks of the disease (e.g., France, Yugoslavia, etc.) formed a well-defined taxon with relatively low variability compared with isolates from Italy where the disease seldom occurs. In another paper, Rekab *et al.* [105] pointed out a polyphyletic nature of this fungus.

Škorić [99] and Dozet [106] reported high levels of resistance to *Phomopsis* in *H. maximiliani*, *H. hirsutus*, *H. pauciflorus*, *H. mollis*, *H. resinosus*, and *H. tuberosus*.

Interspecific hybrids based on *H. eggertii* and *H. smithii* showed high tolerance to *Phomopsis* in Bulgaria [107].

Christov [78] identified annuals *H. annuus*, *H. argophyllus*, and *H. debilis* and perennials *H. pauciflorus*, *H. glaucophyllus*, and *H. eggertii* as potential sources of *Phomopsis* brown stem canker resistance, based on field screening in Bulgaria.

Nikolova *et al.* [108] reported resistance to stem canker in progenies of interspecific hybrids of perennial *H. pumilus*. Resistance to *Phomopsis* was reported in interspecific hybrids derived from *H. argophyllus*, *H. deserticola*, *H. tuberosus*, and *H. xlaetiflorus* [109].

Complete resistance to *Phomopsis* was reported in interspecific hybrids of *H. salicifolius* by Encheva *et al.* [110] and Škorić [22].

State research and private companies have developed a rich germplasm for *Phomopsis* resistance.

### 2.1.5. *Verticillium* wilt (*Verticillium dahliae* Kleb.)

In addition to *Verticillium dahliae* Kleb., sunflowers are attacked by *Verticillium albo-atrum* R. et B. and *Verticillium lateritium* Bertk. *Verticillium dahliae* Kleb. is the most harmful of these three fungi and it is also the most widespread globally. It causes economic damage to sunflower production in North and South America, Europe, North Africa, Australia, and some countries in Asia [22].

Sunflower breeding for *Verticillium* wilt resistance has been extensive in the USA, Canada, and Argentina. Putt [111] discovered the first sources of resistance to *Verticillium* wilt. His discovery was confirmed by Fick and Zimmer [112]. Resistance to the American race was found in the line HA-89 derived from the Russian cultivar VNIIMK 8931. It is controlled by a single dominant gene.

Bruniard *et al.* [113] and Bertero de Romano [114] found a *Verticillium* race in Argentina that could not be controlled by the gene  $V_1$  (HA89).

Bruniard *et al.* [113] reported to have developed the lines V144, V99, V134, and V196 resistant to the Argentine race of *Verticillium*.

Gulya [54] reported that in 2002 he had found a new strain of *V. dahliae*, which was able to overcome the simple,  $V-1$  dominant resistant gene used in oilseed and confection hybrids. The author tested a diverse germplasm and found that the Russian variety VNIIMK 8883 had genes for resistance to the new strain of *Verticillium dahliae*.

Several researchers used wild sunflower species in order to identify the source of resistance to *Verticillium* wilt.

Assessing the resistance of interspecific hybrids (cultivated sunflower  $\times$  *H. tuberosus*) to *Verticillium* wilt, Galina Pustovoit and Krokhin [115] found a different mode of inheritance of resistance (two or three recessive genes or two complementary dominant genes), which hinders the development of resistant genotypes.

Putt [111] discovered a source of resistance in line CM144, which was derived from an interspecific hybrid of wild *H. annuus*. Škorić [116] determined high tolerance to *Verticillium dahliae* in *H. occidentalis*, *H. hirsutus*, and *H. tuberosus*.



### 2.1.6. Charcoal rot [*Macrophomina phaseolina* (Tassi) Goid]

Synonyms for this fungus are *Sclerotium bataticola* Taub., *Macrophomina phaseoli* (Maubl.) Ashby and *Rhizoctonia bataticola* (Taub.) Butler.

Charcoal rot causes economic damage to sunflower production in arid regions. It is widespread in most sunflower-growing countries.

Charcoal rot may cause premature death of sunflowers grown on light, sandy soil under hot and dry climate. The disease is well known in the southern part of Europe [52].

Manici *et al.* [117] concluded that the great variability in pathogenicity in all the climatic areas of Italy suggests good adaptation of *Macrophomina* to the host.

This pathogen has been studied by many authors. Iliescu [118] and Ionita and Iliescu [119] published a detailed review of charcoal rot symptomatology, taxonomy, epidemiology, pathogenesis, and control of *Macrophomina* in sunflowers. To our knowledge, a most detailed description of charcoal rot has been provided by Aćimović [120].

Walcz and Piszkev [121] have developed an inoculation method for screening sunflower lines for resistance to this pathogen.

Mihaljčević [122, 123] conducted the most detailed studies on the effectiveness of inoculation methods with *Macrophomina*. According to his results, the method of Hsi (1961) was the best of the four inoculation methods tested. Hsi developed this method for sorghum testing and Mihaljčević [122] adapted it for sunflower testing.

Ahmad *et al.* [124] examined 13 exotic sunflower inbred lines and eight *Macrophomina* isolates. The tested inbred lines differed significantly in agronomic characteristics (head diameter, head weight, number of seeds per head, 1000-seed weight, and yield per unit area). The inbred lines HAR 1 and HAR 2 were resistant/tolerant across all charcoal rot isolates, while HA 822 was susceptible to the disease development and two charcoal rot isolates (MP9 and MP21) were virulent in affecting the head weight.

Mihaljčević [122] also found high resistance levels in lines derived from the Argentine cultivars Pehuan INTA, Ciro, and Klein as well as in the lines GVP-1 and GVP-2, derived from varietal populations (VNIIMK, Krasnodar) developed by interspecific hybridization with *H. tuberosus*.

Galina Pustovoi and Gubin [83] found the sources of resistance to *Macrophomina* in the F<sub>14</sub> progenies of the interspecific hybrid VNIIMK8931 × *H. tuberosus*. A radical inoculation method (injecting fungus suspension into the head tissue) confirmed a complete resistance in 62 lines.

Studies of wild sunflower species have been insufficient to enable the identification of resistance genes as the sources of resistance against charcoal rot. Seiler [49] concluded that interspecific hybrids based on *H. tuberosus* have resistance to charcoal rot. Wild species *H. mollis*, *H. maximiliani*, *H. resinosus*, *H. tuberosus*, and *H. pauciflorus* have also shown resistance.



#### 2.1.7. *Phoma* black stem (*Phoma macdonaldii* Boerema)

According to Aćimović [120], the synonym for this fungus is *Phoma oleracea* var. *helianthi-tuberosi* Sacc.

*Phoma* black stem is in large expansion in several countries in the world. It causes premature drying of plants (forced ripening) resulting in economic damage that increases from 1 year to another [22].

Viranyi [52] points out that *Phoma* black stem is extremely severe in France where basal stem lesions often result in lodging.

The inoculation method described by Maširević [125] is recommended to sunflower breeders. For efficiency, molecular markers should be used when screening breeding material.

Fayzalla [126] examined in detail the resistance to *Phoma macdonaldii* in a large set of Novi Sad genotypes of cultivated sunflower and several wild species. Using an inoculation method, he found that there was no satisfactory tolerance to *Phoma macdonaldii* in the genotypes of the cultivated sunflower. Among the wild species, however, high tolerance was registered in *H. maximiliani*, *H. argophyllus*, *H. tuberosus*, and *H. pauciflorus*.

*Phoma* black stem resistance has been reported in several perennial species: *H. eggertii*, *H. hirsutus*, *H. resinosus*, and *H. tuberosus* [99].

Encheva *et al.* [110] stated that interspecies of hybrids based on *H. salicifolius* are highly resistant to *Phoma* black stem.

Christov [78] also confirms that interspecies hybrids based on *H. eggertii*, *H. debilis*, and *H. argophyllus* exhibit high levels of resistance to *Phoma*.

Darwishzadeh *et al.* [127] undertook experiments to determine the partial resistance of sunflower genotypes to seven isolates and highly significant differences were observed among genotypes, isolates, and their interactions. Two genotypes exhibited specific resistance with a wide range of isolate-nonspecific partial resistance appearing as well. In addition, QTLs were also found associated with isolate-specific and nonspecific resistance [128]. Alignan *et al.* [129] developed a 1000-element cDNA microarray-containing genes putatively involved in primary metabolic pathways in order to identify genes responsible for partial resistance. They were successful in identifying 38 genes differently expressed among genotypes, treatments, and times.

According to Škorić [99], resistance to *Phoma* black stem is positively correlated with resistance to *Phomopsis* stem canker and charcoal rot.

#### 2.1.8. *Alternaria* blight (*Alternaria helianthi* Tub. et Nish.)

Aćimović [120] cited the following synonyms for *Alternaria* blight: *Helminthosporium helianthi* Hansf., *Alternaria leucanthemum* Nelen et Vas. and *Embellisia helianthi* (Hansf.). The same author stated that sunflowers are also attacked by *Alternaria zinniae* Pape, *Alternaria alternata* (Fr.) Keiss (synonym *Alternaria tenuis* Ness.) and *Alternaria helianthinificiens* Simmons, Walcz,

and Roberts. Of these species, *Alternaria helianthi* is the most common on sunflowers and the best studied from the point of view of sunflower resistance. It was found to attack sunflowers in all continents where this oilseed crop is grown. In the previous decade, it caused the most extensive economic damage on sunflowers in India and Brazil. According to Aćimović [120], most of the cultivated sunflower genotypes are sensitive to *Alternaria* blight.

Regina *et al.* [130] concluded that the occurrence of *Alternaria helianthi* in southern Brazil depended on the pathogen race and sunflower cultivar to a large extent. Attack is most intensive on crops sown in December and least intensive in October-sown crops. Dudienas *et al.* [131] claimed that *Alternaria* causes economic damage in Brazil, especially in humid conditions.

Aćimović [132] tested 1389 inbred lines for 4 years under field conditions and found that only six lines possessed satisfactory tolerance to *Alternaria* blight.

Madhavi *et al.* [133] found the sources of resistance to *Alternaria* blight in *H. tuberosus* and *H. occidentalis*.

Lipps and Herr [134] examined 496 sunflower genotypes for resistance to *Alternaria* for 3 years and found tolerance in eight genotypes only. A different situation was encountered when the *H. tuberosus* population was inoculated in the greenhouse. Based on the obtained results, the authors concluded that *H. tuberosus* can be used as a source of resistance to *Alternaria helianthi*.

Morris *et al.* [135] confirmed that all 21 annual taxa and 18 of 21 perennial species evaluated were susceptible to *A. helianthi* using applied spore suspensions, while perennial species *H. hirsutus*, *H. pauciflorus* ssp. *subrhomboides*, and *H. tuberosus* appear to resist infection by *A. helianthi*.

Sujatha *et al.* [136] determined that nine perennial *Helianthus* species, *H. maximiliani*, *H. mollis*, *H. divaricatus*, *H. simulans*, *H. occidentalis*, *H. pauciflorus* and *H. decapetalus*, *H. resinosus*, and *H. tuberosus* were highly resistant to *Alternaria* leaf spot; all annuals were susceptible.

Christov [78] reported that perennial *H. decapetalus*, *H. laevigatus*, *H. glaucophyllus*, and *H. ciliaris* were potential sources of genes for *Alternaria* resistance.

Complete resistance to *Alternaria* leaf spot was reported in interspecific hybrids of *H. salicifolius* by Encheva *et al.* [110]. Škorić [81] obtained similar results.

## 2.2. Other fungal diseases

There is a large number of other fungal diseases of sunflower that cause economic damage to sunflower production in some regions and in some years. Unfortunately, most of them have not been included in breeding programs yet [22].

### 2.2.1. *Fusarium* wilt (*Fusarium* spp.)

According to Aćimović [120], several species of the genus *Fusarium* attack sunflowers: *Fusarium solani*, *Fusarium solani* var. *minus*, *Fusarium oxysporum*, *Fusarium oxysporum*, *F.*

*helianthi*, *Fusarium moniliforme* (syn. *Gibberella fujikuroi*), *Fusarium equiseti*, *Fusarium tabacum*, *Fusarium culmorum*, *Fusarium* sp. and *Fusarium* spp.

Viranyi [52] states that *Fusarium wilt* (*Fusarium* spp.) has been reported as a pathogen of concern only from Russia [137] where it appeared to be harmful for sunflower production. Based on the extent of necrosis incited by the fungus on the main root and the root–hypocotyl transition zone of sunflower seedlings, some tolerance to pathogen attack could be detected among the genotypes [138]. In a breeding program, a number of new breeding lines were developed exhibiting relatively good field tolerance [139].

There are few research papers dealing with sunflower resistance to *Fusarium*. In one of these earlier papers, Orellana [140] reported that out of 49 inbred lines tested, 23 were resistant to *Fusarium moniliforme*. In recent years, Goncharov [139] produced plants tolerant to *Fusarium* on the basis of laboratory tests and individual selection of plants from three double-cross combinations and an F<sub>3</sub> cross (UV.680 × o.p. cv. Leader).

#### 2.2.2. Rhizopus head rot (*Rhizopus* spp.)

Dry rot of sunflower is caused by the following fungi from the genus *Rhizopus*: *Rhizopus arrhizus* Fisch. (syn. *Rhizopus nodosus* Namysl.), *Rhizopus nigricans* Ehr. (syn. *Rhizopus stolonifer* Eh. et Fr.) and *Rhizopus oryzae* Nent et Geer [120].

Dry rot occurs typically in regions with dry climate and high temperatures. It often causes a significant yield reduction and it particularly reduces the oil content in seeds [22].

It has become an important disease of sunflower in the USA [141]. The disease reduced oil quality and quantity in oilseed sunflower [142]. Infection of sunflower with *Rhizopus* head rot is enhanced by larval feeding of sunflower moth, *Homoeosoma electellum* (Hulst), which contributes to a secondary infection [141].

Yang *et al.* [143] reported that 4 out of 32 wild species and subspecies were resistant when inoculated with *R. arrhizus* and *R. oryzae* Went. The resistant sources were perennial *H. divaricatus*, *H. hirsutus*, *H. xlaetiflorus*, and *H. resinosus*.

One of the pioneer works was that of Agrawat *et al.* [144], who studied the resistance to *Rhizopus nodosus* in 91 sunflower cultivars. Their results based on an inoculation test indicated that resistance existed only in cultivars – Armavirec, Armavirskiy 3497, EC 40277, and K-2217, all from Krasnodar.

*Rhizopus* head rot brings great economic damage in many countries, by decreasing seed yield, seed oil content, and seed development. Unfortunately, few researchers in the world work on the examination of this pathogen.

#### 2.2.3. Powdery mildew (*Erysiphe cichoracearum* DC)

Sunflower is a host to three fungal genera that cause powdery mildew [120, 145]: *Erysiphe cichoracearum* DC, which is widespread in all continents where sunflower are grown, *Leveillula compositarum* Golow., *Leveillula taurica* (Lev.) Arn., and *Sphaerotheca fuliginea* (Schlecht. ex Fr.) Poll.

Since *Erysiphe cichoracearum* DC is common on sunflowers around the world, resistance to this pathogen has been studied most extensively. Saliman *et al.* [146] was among the first to identify wild species from the genus *Helianthus* resistant to *Erysiphe* [120].

Jan and Chandler [147] transferred the resistance from *H. debilis* Nutt. into the line P21. The mode of inheritance in this resistance source was partially dominant. According to unpublished results of Škorić, resistance to powdery mildew exists in several inbred lines, especially those that incorporate genes from *H. tuberosus*.

Breeding programs conducted in Argentina, Australia, and the Republic of South Africa have been targeted on Albugo resistance and several highly tolerant hybrids were obtained.

Seiler [49] indicates that *Helianthus debilis* ssp. *praecox*, and *H. bolanderi*, and 14 perennial species were tolerant of powdery mildew in both field and greenhouse tests [146]. Not all population of perennial species are resistant: population of *H. grosseserratus* and *H. maximiliani* showed differential reactions. Škorić [116] reported that interspecific hybrids with *H. giganteus*, *H. hirsutus*, *H. divaricatus*, and *H. salicifolius* had no powdery mildew symptoms.

Jan and Chandler [147] transferred the resistance from *H. debilis* Nutt. into the line P21. The mode of inheritance in this resistance source was partially dominant. According to unpublished results of Škorić, resistance to powdery mildew exists in several inbred lines, especially those that incorporate genes from *H. tuberosus*.

#### 2.2.4. *Botrytis cinerea* Pers.

Sunflower geneticists and breeders have unjustly neglected the polyphagous fungus *Botrytis cinerea* Pers., although it causes economic damage in sunflower production in some regions.

Prats [148] was the first to discover a source of resistance to *Botrytis cinerea* in the cultivar INRA 64-01.

Burlov and Artemenko [149] found the line Od-2624 to be resistant to *Botrytis*.

Kostyuk [150] studied some 1400 sunflower genotypes and found that none of them were resistant and only some were tolerant to *Botrytis* under natural and inoculation conditions.

#### 2.2.5. White rust (*Albugo tragopogonis* Schr.)

According to Aćimović [120] the synonym for this fungus is *Albugo tragopogonis* (Pers.) Schr. White rust has been registered on sunflowers in several countries and is particularly aggressive in South America (Argentina), Africa (Republic of South Africa), some Asian countries, several countries from the former Soviet Union, and Australia.

Breeding programs conducted in Argentina, Australia, and the Republic of South Africa have been targeted on *Albugo* resistance and several highly tolerant hybrids were obtained [22].

An established breeding centre, which focuses its research on identifying sources of resistance to white rust in wild species of genus *Helianthus*, unfortunately does not exist anywhere in the world.

### 2.3. Viruses and Bacteria

Some viruses are capable of causing disease in sunflowers. The number of viruses that are specific and attack only sunflowers is very limited. In most cases, the main host is another agricultural crop and sunflower is only a secondary host [120]. According to Gulya *et al.* [145], several viruses attack sunflower: aster yellows virus, cucumber mosaic virus, sunflower mosaic virus, sunflower ringspot virus, sunflower yellow blotch virus, leaf crinkle virus, tobacco ringspot virus, tobacco streak virus, tomato spotted wilt virus, potyvirus, etc.

Viruses are typically transmitted by vectors, the most important among which are aphids.

Srechari *et al.* [151] reported three aphid species, *Aphis gossypii* Glove., *Aphis craccivora* Koch, and *Rhopalosiphum maidis* (Fitch), as virus vectors. Among them, *A. gossypii* is best known as the vector that transmits the sunflower mosaic disease.

Lenardon *et al.* [152] detected the sunflower chlorotic mottle virus (SuCMoV) in several regions of Argentina.

Lenardon *et al.* [153] tested 232 lines in the greenhouse using an inoculation method. Only three lines exhibited partial resistance (L33, L74, and L42) to the sunflower chlorotic mottle virus. Of these three lines, L33 was the most resistant. Screening F<sub>2</sub> population of crosses between resistant and sensitive lines in the greenhouse and in field, the authors concluded that the resistance is controlled by a single dominant gene (Remo-1).

In recent years, the sunflower chlorotic mottle virus has been studied intensively at the molecular level.

Dujovny *et al.* [153] conducted a molecular characterization of a new potyvirus (SuCMoV). Arias *et al.* [155] described the effect of SuCMoV on some aspects of carbon metabolism in sunflower plants.

Mailo *et al.* [156] mechanically inoculated one sensitive (20 016) and one tolerant line (B-133) with SuCMoV. Total RNA was isolated from infected leaf tissue for study at the molecular level. The achieved results indicated that the gene expression profiles in the inoculated plants (of the sensitive and the tolerant line) were statistically significant compared with leaves of plants that were not inoculated. Eighty-eight genes were differentially expressed in the tolerant line.

#### 2.3.1. Bacterial diseases

Bacterial diseases of sunflower are caused by pathogenic bacteria. They can be found on sunflowers in most countries where this oil crop is grown. In addition to sunflower, most of these bacteria also attack other crops. The sunflower is typically a secondary host and quite rarely the main host [120].

The most widespread bacteria on sunflowers are *Agrobacterium tumefaciens* (E. F. Sm. and Town.) Conn, *Pseudomonas syringae* pv. *tabaci* (Wolf and Foster 1917) Young, Dye and Wilkie 1978 (synonyms *Pseudomonas tabaci* and *Bacterium tabacum* Wolf and Foster), *Xanthomonas campestris* pv. *phaseoli* (Smith) Due., *Pseudomonas syringae* pv. *helianthi* (Kawamura) Dye, Wilkie

and Young, *Pseudomonas solanacearum* (Smith), *Erwinia carotovora* pv. *carotovora* (Jones) Bergey *et al.*, etc. [120].

There are few papers in the literature dealing with sunflower selection for resistance to bacterial diseases.

Among these few, Nemeth and Walcz [157] reported the occurrence of *Erwinia carotovora* on sunflowers in Hungary in the period 1984–1986. Testes of inbred lines and commercial hybrids conducted under natural conditions indicated that there existed significant differences in resistance to this bacterial disease. However, the tests showed that the breeding material can be tested by inoculation methods under field conditions.

#### 2.4. Sunflower breeding for resistance to broomrape (*Orobanche cumana* Wallr.)

The parasitic angiosperm broomrape (*Orobanche cumana* Wallr. = *Orobanche cernua* Loelf.) is the cause of many economic losses in sunflower production in a number of countries in the world, especially in central and eastern Europe, Spain, Turkey, Israel, Kazakhstan, and China. Its presence has also been established in Australia.

Sunflower breeders have been fighting *Orobanche cumana* Wallr. for almost a century [22].

According to past researches, there have been different mechanisms of sunflower resistance to *Orobanche*. Most often these are genetic mechanisms, but there are also physiological, biochemical, mechanical, and others.

According to Morozov [158], the first reports of broomrape in sunflower came from Saratov Oblast in Russia and date back to the 1890s. The same author mentions that the first sunflower varieties resistant to race A of *Orobanche* were developed by Plachek at the Saratov breeding station.

At the beginning of the 20<sup>th</sup> century, broomrape spread across Russia significantly and endangered the mass production of sunflower. The first cultivar resistant to race A, Saratovskij 169, was created by Plachek. In the years that followed, other cultivars resistant to race A were also produced (Kruglik A/41, Zelenka, and Fuksinka). As the mass production of sunflower spread quickly, it was followed by a relatively fast production of a new race called B. Zhdanov in Rostov on the Don announced that he had produced several cultivars resistant to a new race (B). During the period 1924–1960, Pustovoit in VNIIMK, Krasnodar created highly productive cultivars, which were resistant to race B [22, 158].

In order to attain their breeding goals and identify sources of broomrape resistance, sunflower breeders must develop a breeding strategy, decide on a breeding method, secure the necessary germplasm and differential lines for broomrape race identification, and choose the appropriate inoculation method and molecular marker technique (marker-assisted selection (MAS)) – Škorić [22].

Vrânceanu *et al.* [159] defined a set of differential lines for the evaluation of the composition of broomrape races. Among them was the AD-66 line, which represented a tester line susceptible to all broomrape races. On the other hand, the differential line (cultivar) Kruglik A41 was

used for race A, the Jdanov-8281 cultivar for race B, the Romanian cultivar Record for race C, line S-1348 for race D, and line P-1380-2 for race E. Păcureanu-Joița *et al.* [160] included the line LC-1093 as a tester for race F. Unfortunately, there have been no tester lines for the latest broomrape races [160].

Genes for resistance to broomrape races A, B, C, and D are present in varietal populations of sunflower developed in breeding programs from Krasnodar, Armavir, Odessa, Fundulea, and several other places [149]. Genes that confer resistance to races E, F, G, and the latest ones, on the other hand, have been identified in certain wild species of the genus *Helianthus* and have been incorporated into cultivated sunflower genotypes by interspecific hybridization [161, 162].

Galina Pustovoit [163] and her team made a great contribution in this area by developing sunflower varieties through interspecific hybridization in which *H. tuberosus* was used as the donor of *Or* genes. These varieties were used in the identification of *Or*<sub>4</sub> and *Or*<sub>6</sub> genes.

Fernandez-Martinez *et al.* [164] tested 44 wild sunflower accessions (representing 27 perennial and 4 annual species) and 44 cultivated sunflower accessions, which they raised in a growth chamber and then transplanted to a greenhouse. The material was inoculated with the virulent race F (population SE 296). Most of the perennial species proved fully resistant to race F.

Among the wild annual species, *H. anomalus* and *H. agrestis* were completely resistant, while *H. debilis* ssp. *cucumerifolius* and *H. exilis* segregated with regard to *Orobanche* resistance [164].

Interspecific hybrids based on *H. eggertii* and *H. smithii* showed total resistance to broomrape in Bulgaria [107]. Broomrape resistance to the local race in Bulgaria was reported in *H. divaricatus*, *H. eggertii*, *H. giganteus*, *H. grosseserratus*, *H. glaucophyllus*, *H. mollis*, *H. nuttallii*, *H. pauciflorus* (= *rigidus*), *H. resinosus*, and *H. tuberosus* [107, 165].

Diploid perennial species *H. divaricatus*, *H. giganteus*, *H. glaucophyllus*, *H. grosseserratus*, *H. mollis*, *H. nuttallii*, and *H. smithii* and their interspecific hybrids were resistant to broomrape [67]. Christov [78] reported that several perennial *Helianthus* species showed 100% resistance including *H. tuberosus*, *H. eggertii*, *H. smithii*, *H. pauciflorus*, and *H. strumosus*.

Jan *et al.* [166] crossed the wild sunflower species *H. maximiliani* Schrad., *H. grosseserratus* Mart., and *H. divaricatus* L. with cultivated sunflower and developed four populations (BR1-BR4) resistant to race F in Spain.

Numerous authors in public institutions and private companies use wild sunflower species as donors of genes for resistance to broomrape.

The sources of resistance to broomrape, which have been discovered so far, mostly use the gene of resistance taken from the wild species of the genus *Helianthus*. According to the results obtained so far, there are over 20 wild species of the genus *Helianthus*, which contain the gene of resistance to broomrape [22].

When broomrape occurred, breeders used infested fields for testing selection materials for broomrape resistance in many countries. This method is not reliable enough since in natural conditions infestation with broomrape on those fields is not equally spread, which causes large

experimental errors. A much more reliable method, which is applied on testing resistance, is mixing of broomrape seed (a certain amount) with the selection material in the process of cultivation. In order to accelerate the process of testing the resistance of selection material, there are certain containers used in a greenhouse during the period autumn/winter, which are filled with a mixture of soil and sand filled with broomrape seeds and the seed of sunflower genotypes that are being tested [22].

Panchenko [167] developed a method of testing the selection materials in the greenhouse, which enables simultaneous testing of a great number of lines that are being created. The purpose of this method is preparation of a medium (sterilized soil + sand or perlite) on the tables in the medium. Following that, the appropriate amount of broomrape seed is added and the selection material is cultivated. Within 3–4 weeks after germination, it is possible to make the evaluation of resistance.

In Rustica Prograin Genetique, Grezes-Besset [168] developed a fast method of testing the selection material of sunflower in plastic tubes (a mixture of sand and perlite), which enables a reliable testing of a large number of lines (hybrids) in small space and the cycle lasts about 3 weeks.

However, the most reliable and the most easily applied method of screening breeding materials for broomrape resistance is the use of molecular markers, QTL, RFLP, RAPD, TRAOP, and SSR markers which have been used for this purpose.

Increased use of marker-assisted selection, which gives quick and reliable results, is very positive for sunflower breeding.

The best example of that is the production of hybrids resistant to the imidazolinone group of herbicides, which has proven itself in mass production by cultivating IMI-resistant hybrids followed by controlling broomrape. IMI-resistant hybrids are very important in regions where new races of broomrape have occurred [22].

Dominant genes for resistance to races A, B, C, D, E, and F have been found and incorporated into cultivated sunflower genotypes. In the last 2–3 years, new broomrape populations have been discovered in several countries. None of the existing commercial hybrids resistant to races A, B, C, D, E, and F have proven resistant to these new populations.

## 2.5. Sunflower breeding for insect resistance

Several hundred different species of insects cause infestations in sunflower. However, economic losses are caused only by a few insect species [169]. Some insects transmit several sunflower diseases [170]. *Homoeosoma* spp. are a significant problem in cultivated sunflower on four continents. *Homoeosoma nebulella* (Hubner) infests sunflower in Europe and Asia. In South America, sunflower is infested by *H. heinrichi* (Pastrana), whereas *H. electellum* (Hulst) causes damage to sunflower in Mexico, USA, and Canada.

Cultivar resistance to European sunflower moth (*H. nebulella*) was incorporated in USSR 60–70 years ago through interspecies hybridization of cultivated sunflower and *H. tuberosus* spp. *Purpurellus*, Cockerell [171, 172].



The mechanism of resistance to sunflower moth exists due to the phytomelanin (carbon) layer in the husk. Black hull colour is positively correlated with phytomelanin content in the husk.

North-American species of sunflower moth (*H. electellum*) is far more virulent on cultivated sunflower than *H. nebullella*; hence, resistance breeding to this insect is of great importance in North America.

According to the results of Rogers and Kreitner [173], the presence of phytomelanin in sunflower seed pericarp prevents seed infestation with *H. electellum*. By monitoring the formation of pericarp (husk), it was determined that phytomelanin starts to accumulate between the hypodermis and sclerenchyma 3 days after fertilization, whereas its formation is clearly visible 13 days after fertilization.

In the major production area of North America, there are about 14 principal insect pests of cultivated sunflower, and of this total about six are considered potential economic pests [174].

“Two breeding procedures are recommended for identifying lines with improved resistance to insects attacking cultivated sunflower. These procedures are based on the initial evidence that the resistance to the insects is quantitatively inherited, that is, controlled by several genes. Both are based on recurrent selection and random mating, with the main objective to combine as many of the alleles controlling resistance as possible” [175].

“The recurrent phenotypic selection breeding procedure could be utilized for selection against stem and/or foliage infesting insects. An original ( $C_0$ ) source population may be created by random mating cultivars or lines (e.g., Plant introductions, open-pollinated populations), which are then screened for resistance to a particular insect attack sunflower” [175].

“Recurrent phenotypic selection with  $S_1$  line progeny evaluation could be utilized for selection for head and/or seed infesting insects. An original ( $C_0$ ) source population is created similarly as in the recurrent phenotypic selection procedure. The  $C_0$  population is planted in a normal breeding nursery with the most vigorous plants selected for bagging and self-pollination” [175].

According to Seiler [49], the insects causing most economic damage in North America are: sunflower beetle [*Zygogramma exclamationis* (Fabritius)], the sunflower stem weevil [*Cylindrocopturus adspersus* (LeConte)], the red and gray seed weevils [*Smicronyx fulvus* (LeConte) and *S. sordidus* (LeConte)], the banded sunflower moth [*Cochylis hospes* Walsingham), the sunflower moth [*Homoeosoma electellum* (Hulst)], and the sunflower midge *Contarinia schulzi* Gagne. The sunflower head moth, *Homoeosoma electellum* is the most widespread and damaging sunflower insect pest in North America, while in Europe and Asia it is *Homoeosoma nebullella* (Hubner).

According to the results obtained by Rogers [176, 177], the following sunflower species exhibit significant levels of resistance to sunflower moth: *H. arizonensis*, *H. ciliaris*, *H. pumilus*, *H. resinosus*, *H. rigidus* × *laetiflorus*, *H. silphoides* and *H. smithii*.

Among the insects that cause economic losses to sunflower production, the biggest success was achieved in breeding for resistance to sunflower head moth above all in Europe by the development of cultivars in which an armored layer was induced in the husk from some specific wild species. Similar results were obtained in North America.

High level of resistance to *Bothynus gibbosus* was exhibited by the following species: *H. tuberosus*, *H. maximiliani*, *H. niveus*, *H. xlaetiflorus*, *H. salicifolius*, *H. mollis*, *H. grosseserratus*, *H. Argophyllus*, and *H. ciliaris* [178].

The results of Rogers [176, 179, 180], as well as Rogers and Thompson [178, 181, 182], confirm high levels of resistance to *Zygogramma exclamationis*, *Bothynus gibbosus*, *Masonaphis masoni* and *Empoasca abrupta* in wild sunflower species *H. tuberosus* and *H. maximiliani*, and recommend the use of these species in breeding programs.

Results of Rogers and Thompson [183, 184] confirm significant levels of resistance in two annual and 10 perennial wild species (*Masonaphis masoni*). The highest resistance to aphids was seen in *H. carnosus*, *H. exilis*, *H. floridanus* and *H. radula*.

Weak point in sunflower breeding for resistance to insects is that only few sunflower researchers deal with this issue. Insecticides can be used to some extent, more or less successfully, as a solution to this problem in some species.

## 2.6. Conclusions

Biotic stresses cause great economic damages and act as a limiting factor for the production of sunflower.

Diseases are the main problem among biotic stresses. Using wild sunflower species of the *Helianthus* genus, genes conferring resistance to most dominant diseases were discovered and incorporated to the genotypes of the cultivated sunflower.

Regarding the achievements in sunflower breeding for disease resistance, the results can be divided into four different groups.

The first group consists of work that resulted in the discovery of genetic resistance to certain causative agents of sunflower diseases (*Plasmopara halstedii*, *Puccinia helianthi*, *Verticillium dahliae*, *Verticillium albo-atrum*, and *Erysiphe cichoracearum*).

The second group comprises work in which a high level of tolerance (field resistance) was achieved. This group includes the results achieved in breeding for resistance to *Phomopsis*/*Diaporthe helianthi*, *Macrophomina phaseolina*, *Albugo tragopogonis*, and *Alternaria* ssp.

The third group consists of results in which a satisfactory level of tolerance was achieved (*Phoma macdonaldii* and to some extent *Sclerotinia sclerotiorum*).

The fourth group consists of results that were partly achieved, where the level of favorable tolerance, that is, resistance, was not reached (*Rhizopus* ssp., *Botrytis cinerea* and other fungal pathogens).

Viruses and bacteria only pose a minor problem in comparison with diseases. Breeding for resistance to viruses and bacteria also includes wild sunflower species.

Broomrape (*Orobancha cumana* Walr.) is a major global issue in sunflower production, particularly in Central and Eastern Europe. Genes conferring resistance to six races of broomrape have been discovered in some wild sunflower species and incorporated into genotypes of the

cultivated sunflower. Research work, which aims at finding genes of resistance to the newest races within specific wild sunflower species, is in progress.

Sources of resistance to imidazolines and sulfonylurea herbicides (tribenuron-methyl) have been found in a population of wild *H. annuus* from Kansas and incorporated in cultivated sunflower genotypes. Moreover, genes conferring resistance to these herbicides were discovered using induced mutations. The newly developed hybrids, resistant to the abovementioned herbicides, provide successful weed and broomrape control through imidazolines.

Insects are a major issue in sunflower production, especially in North America. Significant results have been obtained through breeding for resistance to sunflower head moth. Wild sunflower species are used in research work aimed at finding the sources of resistance to other economically harmful insects.

Different biotechnological methods (tissue culture, embryo culture, protoplast fusion, molecular markers, in vitro screening, and other methods) have been included in breeding for resistance to biotic stresses.

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## Evolution and Adaptation in Stress Tolerance

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# Drought Adaptation in Millets

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Additional information is available at the end of the chapter

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## Abstract

Millets are major food and feed sources in the developing world especially in the semi-arid tropical regions of Africa and Asia. The most widely cultivated millets are pearl millet [*Pennisetum glaucum* (L.) R. Br.], finger millet [*Eleusine coracana* (L.) Gaertn], foxtail millet [*Setaria italica* (L.) P. Beauvois], Japanese barnyard millet [*Echinochloa esculneta* (A. Braun) H. Scholz], Indian Barnyard millet [*Echinochloa frumentacea* Link], kodo millet [*Paspalum scrobiculatum* L.], little millet [*Panicum sumatrense* Roth.ex.Roem. & Schult.], proso millet [*Panicum miliaceum* L.], tef [*Eragrostis tef* (Zucc.) Trotter] and fonio or acha [*Digitaria exilis* (Kippist) Stapf and *D. iburua* Stapf]. Millets are resilient to extreme environmental conditions especially to inadequate moisture and are rich in nutrients. Millets are also considered to be a healthy food, mainly due to the lack of gluten (a substance that causes coeliac disease) in their grain. Despite these agronomic, nutritional and health-related benefits, millets produce very low yield compared to major cereals such as wheat and rice. This extremely low productivity is related to the challenging environment in which they are extensively cultivated and to the little research investment in these crops. Recently, several national and international initiatives have begun to support the improvement of diverse millet types.

**Keywords:** Abiotic stress, drought avoidance, drought escape, drought tolerance, millet

## 1. Introduction

Millets are among the major cereal crops in the developing world especially in the semi-arid tropical regions of Africa and Asia where they are used both as human food and livestock feed. Millets represent small grain crops that are mainly cultivated in marginal environments. Exceptional to this definition is pearl millet [*Pennisetum glaucum* (L.) R. Br.] that has a large seed size. Among the widely cultivated millets, those traditionally considered as millet are pearl millet, finger millet [*Eleusine coracana* (L.) Gaertn], foxtail millet [*Setaria italica* (L.) P. Beauvois], Japanese barnyard millet [*Echinochloa esculneta* (A. Braun) H. Scholz], Indian

Barnyard millet [*Echinochloa frumetacea* Link], kodo millet [*Paspalum scrobiculatum* L.], little millet [*Panicum sumatrense* Roth ex Roem. & Schult.] and proso millet [*Panicum miliaceum* L.] (Table 1). Tef [*Eragrostis tef* (Zucc.) Trotter] was included in the millet group at the First Small Millets Workshop held 30 years ago in Bangalore, India [1], while both tef and fonio or acha [*Digitaria exilis* (Kippist) Stapf and *D. iburua* Stapf] were grouped under small millets by international agricultural organizations in the mid-1990s [2]. The inclusion of tef and fonio to the millet family is justifiable due to the close relationship of the two species with other millets. The genetic difference between some traditional millets is as large as that between tef or fonio and other millets. Due to this substantial variability among themselves, millets are grouped into two subfamilies, namely Panicoideae, which includes pearl millet, foxtail millets, Japanese barnyard millet and Indian millet, and to Chloridoideae, which includes finger millet and tef, and eight genera (Table 1). This indicates that finger millet which is normally grouped under millet is more closely related to tef than to other millets [3]. The divergence among traditional millets is also exhibited in the chromosome number and ploidy level which range from the diploid pearl millet ( $2n = 2x = 14$ ) to the hexaploid fonio ( $2n = 6x = 54$ ) [4, 5]. Millets are also divergent in the size and colour of seeds, seed weight, plant stature and shape of their panicles (Table 1). The geographical distributions of small millets were recently summarized by Goron and Raizada [6]. Except for finger millet, which is extensively cultivated in Africa and Asia, other small millets are mainly grown in Asia.

Common name	Traditional millets				
	Pearl millet	Foxtail millet	Japanese Barnyard millet	Indian Barnyard millet	Kodo millet
Other names	Bulrush millet	Italian millet	Japanese millet	Billion dollar grass	Koda millet
Botanical names	<i>Pennisetum glaucum</i>	<i>Setaria italica</i>	<i>Echinochloa esculneta</i>	<i>Echinochloa frumetacea</i>	<i>Paspalum scrobiculatum</i>
Subfamily	Panicoideae	Panicoideae	Panicoideae	Panicoideae	Panicoideae
Tribe	Paniceae	Paniceae	Paniceae	Paniceae	Paniceae
Distribution			Japan, Korea, China	India, Pakistan, Nepal	
Ploidy level	Diploid	Diploid	Hexaploid	Hexaploid	Tetraploid
Chromosome number	$2n = 2x = 14$	$2n = 2x = 18$	$2n = 6x = 36$	$2n = 6x = 36$	$2n = 4x = 40$
Purpose	Food, feed	Food, biofuel	Food, feed	Food	Food, feed
Agronomic benefits	Drought & heat tolerance	Drought tolerance	Early maturity, anti-fungal	Early maturity	Drought tolerance
Nutritional benefits	High protein, starch & minerals		anti-diabetic	High protein content	High-quality protein
Health benefits	No gluten	No gluten	No gluten	No gluten	Low glycaemic index, anti-oxidant
Reference	[4, 20, 27]	[16, 27]	[5, 18, 27]	[6]	[27, 36]



Common name	Traditional millets			Other millets	
	Little millet	Proso millet	Finger millet	Tef	Fonio
Other names		Common millet	Ragi, African millet	Teff, lovegrass	Acha
Botanical names	<i>Panicum sumatrense</i>	<i>Panicum miliaceum</i>	<i>Eleusine coracana</i>	<i>Eragrostis tef</i>	<i>Digitaria exilis</i> , <i>D. Iburua</i>
Subfamily	Panicoideae	Panicoideae	Chloridoideae	Chloridoideae	Panicoideae
Tribe	Paniceae	Paniceae	Eragrostideae	Eragrostideae	Paniceae
Distribution					
Ploidy level	Tetraploid	Tetraploid	Tetraploid	Tetraploid	Diploid or hexaploid
Chromosome number	$2n = 4x = 36$	$2n = 4x = 36$	$2n = 4x = 36$	$2n = 4x = 40$	$2n = 2x = 30$ or $2n = 6x = 54$
Purpose	Food	Food, feed	Food, feed	Food, feed	Food, feed
Agronomic benefits	Abiotic stress tolerance	Drought tolerance, early maturity	Drought and salt tolerance	Water-logging tolerance, storage pest tolerance	Drought tolerance
Nutritional or	High in phytochemicals, fibre	Rich in amino acids	Rich in calcium, methionine and tryptophan	Rich in protein	Rich in amino acids
Health benefits	Anti-diabetic	Anti-cancer	Low glycaemic index, anti-oxidant	No gluten	
Reference	[38–40]	[6, 23, 27]	[6, 20, 27, 35, 37]	[33, 47]	[20, 24, 25]

**Table 1.** Description and benefits of millets

## 2. Importance of millets in global agriculture

### 2.1. Economic benefits

Millets play a key role in the economy of the developing world especially in countries with extensive areas of marginal land used for crop cultivation. In 2013, the global area under millet cultivation was 34.9 million hectares, corresponding to 4.7 % of the global area for all cereals including wheat, maize and rice [7] (Table 2). On the other hand, the global production of millets in the same year was estimated to be 36.7 million tons, which contributes only 1.2% to the total cereal production. This lower production was due to the inferior average yield of millets (only 0.9 t ha<sup>-1</sup>) compared to other cereals (3.8 t ha<sup>-1</sup>). However, the contribution of India to global millet production is significant. In 2013, India produced over 30% of the global millet yield from only 25% of the global millet area, mainly due to improved productivity. In the same year, while the mean seed yield of millet in India was 1.2 t ha<sup>-1</sup>, it was only 0.8 t ha<sup>-1</sup> for other countries. This 50% production advantage in India over other countries especially African countries was due to the widespread use of improved varieties and techniques. A

decade ago, the rate of adoption of improved pearl millet cultivars by farmers was 65% in India but below 10% in some African countries [8].

Country	Traditional millets <sup>a</sup>	Tef	Fonio (Acha)	Total
<b>Production (ton)</b>				
India	10,910,000			10,910,000
Ethiopia	848,956 <sup>b</sup>	4,418,642		5,267,598
Nigeria	5,000,000		90,000	5,090,000
Niger	2,995,000		6,000	3,001,000
China	1,746,000			1,746,000
Mali	1,152,331		22,090	1,174,421
Burkina Faso	1,078,570		19,887	1,098,457
Sudan (former)	1,090,000			1,090,000
Guinea	215,000		429,000	644,000
Chad	582,000			582,000
Senegal	572,155		1,030	573,185
Russia	418,844			418,844
USA	418,145			418,145
Tanzania	322,731			322,731
Pakistan	310,000			310,000
Nepal	305,588			305,588
Uganda	228,000			228,000
Myanmar	185,000			185,000
Ghana	155,131			155,131
Cameroon	97,000			97,000
others	1,233,696		19,000	1,252,696
Total production	29,864,147	4,418,642 <sup>c</sup>	587,007	34,869,796
<b>Total area (ha)</b>	33,118,792	3,016,521	554,451	36,689,764
<b>Yield (ton ha<sup>-1</sup>)<sup>d</sup></b>	0.90	1.47	1.06	0.95
Reference	[7,9]	[9]	[7]	

<sup>a</sup> Traditional millets include finger millet, foxtail millet, Indian barnyard millet, Japanese barnyard millet, kodo millet, little millet, pearl millet and proso millet.

<sup>b</sup> Only for finger millet.

<sup>c</sup> Only for Ethiopia.

<sup>d</sup> Average global yield except for tef where it is the national average yield for Ethiopia.

**Table 2.** The top 20 millet-producing countries in the world in 2013.

Tef and fonio are exclusively cultivated in Africa. While fonio is cultivated on a total of half a million hectares in West Africa mainly in Guinea, Guinea-Bissau and Côte d'Ivoire [7], tef is grown in the Horn of Africa especially in Ethiopia where it is annually cultivated on three million hectares of land and is a staple food for about 50 million people [9]. In the last two decades, the productivity of tef was raised by 100%, from just 0.7 t ha<sup>-1</sup> in 1994 to 1.4 t ha<sup>-1</sup> in 2013 mainly due to an increase in the use of improved cultivars.

In general, millets play a key role in food security in Asia and Africa. Together with sorghum, millets account for about half of the total cereal production in Africa [10]. Millets, are therefore considered as a poor man's crop due to their significant contributions to the diet of resource-limited farmers and consumers.

## 2.2. Agronomic benefits

Millets are resilient to the extreme climatic and soil conditions prevalent in the semi-arid regions of Asia and Africa. The similarities of millets are that they are all grown under extreme environmental conditions, especially those of inadequate moisture and poor soil fertility which are poorly suited to the major crops of the world [11] (Table 1). Proso millet is considered to have been domesticated before rice in China, based on the extreme resistance of this millet to drought [12, 13]. In addition to its resistance to drought, proso millet escapes the terminal drought that normally occurs late in the growing season since it matures in only three months; hence, proso millet is considered to be a millet with low water requirements [6].

Similar to maize and sorghum, millets possess a C<sub>4</sub> photosynthesis system [14, 15]; hence, they prevent photorespiration and, as a consequence, efficiently utilize the scarce moisture present in the semi-arid regions. Since C<sub>4</sub> plants are able to close their stomata for long periods, they can significantly reduce moisture loss through the leaves. In addition to its tolerance to drought, tef is tolerant to waterlogging especially in poorly drained soils where other crops such as maize and wheat could not survive. Foxtail millet is also considered to be a model plant for biofuel studies [16]. A novel peptide isolated from foxtail millet and barnyard millet has shown strong antifungal properties as has one from finger millet which is especially effective and works against four fungus species, namely *Alternaria*, *Trichoderma*, *Botrytis* and *Fusarium* [17, 18].

## 2.3. Nutritional benefits

Millets are rich sources of nutrients for both humans and animals. Saleh et al. [19] have compiled detailed information on the nutritional advantages of several millets. The grains of most millets possess levels of protein comparable to those of wheat but higher than those of rice [20] (Table 1). In addition, the seeds of finger millet contain valuable amino acids especially methionine [20], which is lacking in the diets of hundreds of millions of the poor who live on starchy staples such as cassava. Other reports indicate that finger millet is rich in lysine, threonine and valine [21, 22] while proso millet has plentiful leucine, isoleucine and methionine [23]. The seeds of fonio are also nutritious, especially in amino acids such as leucine, methionine and valine [24, 25]. Since proso millet is rich in essential amino acids

including leucine, isoleucine and methionine, the protein quality of the grain is higher than that of wheat [23].

The grains of extensively cultivated pearl millet contain high amounts of starch, fibres and minerals [26, 27]. In general, millets have high amounts of vitamins, calcium, iron, potassium, magnesium and zinc [28].

The straws and crop residues of millets are also the main source of livestock feed for farmers in developing countries. In Ethiopia, compared to the straw from other cereals, the straw of tef is the most palatable to livestock and fetches the highest price [29].

#### **2.4. Health-related benefits**

In addition to being nutritious, millets are also considered to be a healthy food. Two recent reviews examined the health-related benefits associated with millets [19, 6]. A number of leading newspapers and media have recently indicated the potential of millets particularly tef as a global lifestyle crop [30–32]. This is particularly due to the lack of gluten in the grain of tef [33] (Table 1). Gluten is a substance present in wheat and other grains that causes celiac disease or other forms of allergies. Similar to tef, several other millets, particularly foxtail millet, do not contain gluten.

Six millet species (namely kodo, finger, proso, foxtail, little and pearl millets) were shown to have an anti-proliferative property and might have a potential in the prevention of cancer initiation [34, 35]. The anti-proliferative property of these millets is associated with the presence of phenolic extracts. Among the first four millets indicated above, the maximum phenolic content was obtained in kodo millet while the minimum was in foxtail millet [36].

Finger millet is also a popular food among diabetic patients because of its low glycaemic index and slow digestion due to high fibre content [37]. The glycaemic index of little millet was also lower than that of rice, wheat and sorghum; hence, it is considered to be an anti-diabetic grain [38]. The composition of useful antioxidants and related products could be enhanced through processing the grain. A study in little millet showed that the levels of phenolics, flavonoids and tannins were substantially increased by germinating, steaming and roasting soaked grains [39].

### **3. Drought: A major challenge to millet cultivation**

Biotic stresses such as insect pests and diseases are a cause for substantial yield losses to diverse types of millets. However, abiotic stresses are the biggest contributor to losses every year. Although, in general, millets perform better than cereals such as wheat and rice in semi-arid environments, these challenging climatic and soil conditions are by no means an optimum environment for millet cultivation. In semi-arid and arid environments where millets are the dominant crop, drought or inadequate moisture is the major abiotic stress affecting productivity. Studies in pearl millet showed that drought impacts include growth, yield, membrane integrity, pigment, osmotic adjustment, water relations and photosynthetic activity [40].

### 3.1. Prevalence of drought

Drought is defined as a temporary reduction in moisture availability in which the amount of available water is significantly below normal for a specified period. In general, drought can be explained as meteorological, hydrological or agricultural drought [41]. Agricultural drought occurs when there is not enough soil moisture to meet the needs of a particular crop at a particular time. Drought is also commonly expressed as a shortage or absence of rainfall causing a loss in rain-fed agriculture. For example, the decline in the level of rainfall during severe drought years in Ethiopia was accompanied by serious reductions in rain-fed agricultural outputs; this is because a 10% drop in rainfall (below the long-term national averages) results in an average drop of 4.2% in cereal yields [42].

As indicated above, millets are crops of dry land areas of the world. According to the United Nations, dry lands, which cover 40% of the world's land area or one-third of the global arable land, support two billion people, of which 90% live in the developing world [43]. Dry lands are classified into four, namely hyper-arid deserts, arid, semi-arid and dry subhumid. Millets are extensively cultivated in the semi-arid region, which is characterized by low and erratic rainfall and periodic drought. Climate change is expected to worsen the situation in this part of the world by reducing the grassland productivity by 49–90% by 2020 [43]. The Sahel Region in Africa, covering over three million km<sup>2</sup> in 10 countries (namely northern Senegal, southern Mauritania, central Mali, northern Burkina Faso, the extreme south of Algeria, Niger, the extreme north of Nigeria, central Chad, central and southern Sudan and northern Eritrea) is the typical semi-arid region situated between the Sahara desert in the north and the tropical or savanna climate in the south [44].

The frequency and intensity of drought has increased in recent times. In Ethiopia, severe droughts used to occur periodically every 6–8 years [45], but recently, they have happened every 1–2 years especially in the south of the country [46].

Similar to other millets, drought is implicated among the major yield limiting factors in tef production [47]. Although tef grows in a wide variety of agro-ecological conditions ranging from semi-arid areas with low rainfall to areas with high rainfall, the rainfall pattern in most tef growing regions is not consistent enough to support the normal growth of the crop during the crop cycle. In most tef growing regions, greater rainfall variability exists over the growing period than over the year-cycle [48, 49] which results in poor agricultural outputs. The Water Requirement Satisfaction Index (WRSI), a crop-specific performance indicator taking rainfall and soil characteristics into account, indicates extreme and increasing variability in Ethiopia. A recent study also confirmed that climate will have a negative impact on the acreage and productivity of tef unless urgent interventions are implemented which favours mitigation and adaptation strategies [50].

### 3.2. Yield losses due to drought

Various yield loss studies made for millets treated with drought conditions are summarized in Table 3. Using polyvinylchloride (PVC) tubes filled with sandy soil, Matsuura and colleagues [51] investigated the effect of moisture deficit before and after flowering on four millets,

namely proso millet, little millet, foxtail millet and wild millet [*Setaria glauca* (L.) Beauv.]. Compared to the well-watered plants, a significant yield reduction was obtained in all four millets when the drought treatment was implemented at early developmental stage, that is, before flowering (or heading). However, terminal drought, which occurs from the flowering stage to the harvesting of the crop, contributed to a significant yield loss only in proso and little millets while the effect on foxtail and wild millets was negligible.

Millet type	Yield loss (%)			Critical stage	Reference
	Early drought <sup>a</sup>	terminal drought <sup>b</sup>	Long-term drought <sup>c</sup>		
Proso millet	30.1*	34.6*	64.0*	Before and after heading	[51]
Little millet	62.6*	80.1*	80.5*		[51]
Foxtail millet	19.2*	3.4 <sup>NS</sup>	20.3*	Before heading	[51]
Wild millet ( <i>Setaria glauca</i> )	27.3*	15.3 <sup>NS</sup>	30.1*		[51]
	Mid-season stress <sup>d</sup>	Terminal stress <sup>e</sup>			
Pearl millet	6.6	60.1		Flowering	[53]
Finger millet		109.8 <sup>f</sup>		Flowering	[54]
	Prior to flowering	Beginning flowering	End of flowering		
Pearl millet	72	61	Insignificant	From four weeks to flowering	[52]
	Early stress <sup>g</sup>				
Tef	69–77				[55]

<sup>a</sup> Early drought: water stress from 25 days after sowing till flowering.

<sup>\*</sup>Indicates statistically significant difference from the well-watered samples.

<sup>b</sup> Terminal drought: water stress from flowering till harvesting.

<sup>c</sup> Long-term drought: water stress from 25 days after sowing till harvesting.

<sup>d</sup> Mid-season stress: water stress for 30 days from floral initiation to flowering.

<sup>e</sup>Terminal stress: water stress at flowering.

<sup>f</sup> Water stress from 28 days after sowing to harvest.

<sup>g</sup> Early stress: water stress from two weeks after emergence until symptoms of stress observed.

**Table 3.** The magnitude of yield loss due to moisture scarcity in millets.

A study by Winkel et al. [52] in Niger where the annual rainfall is around 200 mm investigated the impact of water deficit at three stages of pearl millet development. The three stages were prior to flowering, at flowering and at the end of flowering. According to the findings of the work, the grain yield of pearl millet was severely reduced when moisture was limited prior to

and at the flowering stage but not at the end of flowering. On the other hand, in pearl millet, terminal drought in which irrigation was terminated from the flowering until crop maturity, was severe, as it resulted in 60% yield loss [53]. The mid-season stress, which occurred from one month before flower initiation to full flowering, resulted in only 7% yield loss.

The study in two landraces of finger millet in which a drought treatment was imposed four weeks after sowing, resulted in 100% yield loss and over 30% biomass damage [54]. Similarly, yield loss reached up to 77% when the tef plant experienced drought at the flowering stage [55].

Although yield loss studies were not exhaustively made for most millets as they are considered drought tolerant, substantial damage occurs to these crops depending on the severity of drought. However, millets produce at least some grain and straw even in bad years unlike drought-intolerant cereals such as wheat and rice which completely fail to produce any yield.

## 4. Adaptation of millets to drought

### 4.1. Strategies to drought adaptation or tolerance

Plants cope with drought using three main strategies, namely, drought escape, drought avoidance and drought tolerance, although a fourth strategy, known as drought recovery, has also been identified [56–60].

**Drought escape:** Drought escape refers to the condition in which plants reach maturity before the drought occurs. Traits associated with drought escape are rapid growth, early flowering, high leaf nitrogen level and high photosynthetic capacity [58]. The study in West Africa indicated that pearl millet matches its phenology to the mean distribution of the rainfall where precipitation is limited and erratic [61]. In this case, the development of the main panicle coincided with an increasing period of rain, thus reducing the risks associated with drought events occurring prior to or at the beginning of flowering.

**Drought avoidance:** Drought avoidance refers to the ability of the plant to maintain a favourable water balance under moisture stress in order to avoid water deficit in the plant tissue. Two types of drought avoidance mechanisms have been identified: (i) those that reduce water loss through transpiration (e.g. low stomata conductance and reduced leaf) and (ii) those that maintain water uptake during drought period (e.g. high root-to-shoot ratio) [56, 58, 62].

**Drought tolerance:** Drought tolerance refers to the ability of the plant to produce some yield by withstanding low water potential [62]. Traits associated with drought tolerance are increased osmoprotectants (or compatible solutes such as betaines and amino acids), and osmotic adjustment (i.e. reducing osmotic potential through accumulation of organic and inorganic substances) [58, 60].

**Drought recovery:** Drought recovery refers to a condition in which plants recover from the adverse effects of drought in order to provide some yield and/or biomass. Desiccation-tolerant or resurrection plants particularly the wild *Eragrostis nindensis* is the typical example of drought recovery since it stabilizes its cells or membranes at desiccated state [63].

These strategies which are devised by plants to cope with drought are manifested through changes in some phenotypic traits. In a recent review, Kooyers [58] showed for each strategy the path followed by plants in terms of life cycle, altered phenotypes and to the type of drought the plant fits itself. This indicates that the strategies and mechanisms of drought tolerance are interrelated.

#### 4.2. Mechanism of drought tolerance

Table 4 summarizes various mechanisms of drought tolerance in diverse millet types. These inherent properties of plants which include agronomical, morphological and physiological traits are briefly discussed below.

Parameter	Millet type	Response to drought	Reference
<b>Agronomy-related traits</b>			
Seed number and biomass	Pearl millet	Unaffected under drought	[64]
Seed yield	Pearl millet	High for drought-tolerant genotypes	[65]
Flowering time	Pearl millet	Adjust phenology to rainfall pattern	[53]
<b>Morphology-related traits</b>			
Shoot length	Little millet	Decreased under drought	[40]
Root length	Little millet	Increased under drought	[40]
Leaf tensile strength	Tef	Increased in drought-tolerant plants	[68]
<b>Physiology-related traits</b>			
Water extraction	Pearl millet	Less extraction before flowering; more extraction after flowering	[65]
Chlorophyll content	Little millet	Decreased under drought	[40]
<b>Biochemical-related traits</b>			
Anti-oxidants	Little millet	Accumulated under drought	[40]
ROS scavenging enzymes	Little millet, tef	Accumulated under drought	[40, 71]
Free proline	Tef, little millet	Increased concentration	[40, 71]
GB (glycine betaine)	Little millet	Accumulated under drought	[40]
Superoxide	Little millet	Accumulated under drought	[40]
AP (ascorbate peroxidase)	Tef, little millet	Increased specific activity	[40, 71]
CAT (catalase)	Little millet	Accumulated under drought	[40]
GR (glutathione reductase)	Tef	Increased concentration	[71]
MDAR (monodehydro-ascorbate reductase)	Tef	Increased concentration	[71]
Total free amino acid	Little millet	Increased concentration	[40]

**Table 4.** Traits associated to diverse drought tolerance mechanisms in millets.



**Agroonomy-related traits:** These refer to the traits that are commonly known as yield and yield components. Among these, number of tillers, number and size of panicle, seed and biomass yield, seed weight and harvest index are the major ones. However, conclusions could not be made from the two studies using drought-tolerant pearl millet cultivars since drought did not affect the shoot biomass in the first case [64] while it boosted the seed yield in the second case [65].

**Morphology-related traits:** Morphological or anatomical traits which play important roles in drought tolerance include root- and shoot length and leaf area [66]. However, changes in the morphological and biochemical properties of the flag leaf play a key role in drought tolerance as flag leaves are the primary source of photosynthesis [67]. Mechanical properties of the plant also affect drought tolerance in millets. Balsamo et al. [68] studied the leaf tensile strength or also known as force to tear in three *Eragrostis* species with different levels of tolerance to drought. According to their findings, drought-tolerant *E. curvula* had higher tensile strength values than the moderately drought-tolerant *E. tef*, which in turn had higher values than the drought-susceptible *E. capensis*, indicating a positive correlation between drought tolerance and leaf tensile strength [68]. Structural investigations of leaves from the three species revealed the presence of extensive lignification of bundle sheath extensions in *E. tef* and *E. curvula* unlike in *E. capensis*. A study in maize indicated that lignification of the midrib parenchyma and epidermis was directly correlated with increased tensile strength [69].

**Physiology-related traits:** Among the several physiological traits that are differentially regulated during moisture deficit, osmotic adjustment is a major mechanism that increases drought avoidance to enable the plant produce some yield. Osmotic adjustment, which refers to the lowering of the osmotic potential in the cytoplasm due to the accumulation of compatible solutes such as proline, glycine betaine and organic acids, contributes to turgor maintenance of shoots and roots [40]. In little millet, drought stress increased the amount of proline and glycine betaine in both the root and leaf [40]. According to the authors, the accumulation of free amino acids in this millet during drought might be related to the disruption in protein synthesis, induced proteolysis or its partial hydrolysis [40]. Water-use efficiency of the plant is also important as moisture is mostly limited in the areas where millets are extensively cultivated. The experiment using drought-sensitive and drought-tolerant pearl millet genotypes showed that under moisture deficit conditions, the total amount of water extracted by both genotypes was comparable [65]. However, compared to susceptible genotypes, tolerant genotypes extracted less water prior to flowering and more water after flowering, enabling these genotypes to support the tillers and maintain the stay-green phenotype.

**Biochemical-related traits:** Reactive oxygen species (ROS) are chemically reactive molecules that are useful in cell signalling at low concentrations but are damaging to cells when present at high concentrations. The main causes for the high production of ROS are environmental stresses such as drought and salinity [70]. In order to reduce the damaging effects of ROS, plants produce antioxidants, which include glutathione, ascorbate and carotenoids and ROS-scavenging enzymes which include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (AP or APX) [40]. In little millet, the activity of SOD, POD and CAT were elevated under drought conditions to enable the plant cope with unfavourable ROS accumulation [40]. Similarly, the activity of AP and monodehydro-ascorbate reductase (MDAR) increased in *tef* plants treated with drought compared to control plants receiving normal watering [71].

4.3. Genes involved in drought tolerance

The sequence of the genome and transcriptome of plants provides information important to the understanding of the types of genes involved in the regulation of drought tolerance, particularly in plants with increased resistant to moisture scarcity. So far, the genome of foxtail millet [72, 73] and tef [3] has been sequenced.

Transcriptome sequencing of millets after exposure to moisture-deficit condition provides information on genes differentially regulated under exposure to abiotic stresses particularly to drought. A transcriptome-wide study of finger millet plants exposed to drought obtained 2824 genes that were differentially expressed under these conditions [74].

Genes known to be involved in drought response and/or tolerance of selected millets are presented in Table 5. Wang et al. [75] indicated that the overexpression of SiLEA14, a type of LEA gene from foxtail millet, increased the tolerance of *Arabidopsis* plants to salt and osmotic stress. Parvathi et al. [76] reported the induction of several genes when finger millet was exposed to drought. The up-regulated genes include metallothionein, farnesylated protein ATPF6, Farnesyl pyrophosphate synthase and protein phosphatase 2A.

Gene name	Source of the gene	Test organism (type)	Reference
SiLEA	Foxtail millet	Overexpression in foxtail millet and Arabidopsis increased drought tolerance	[75]
SiARDP	Foxtail millet	Overexpression in foxtail millet and Arabidopsis increased drought tolerance	[83]
EcDehydrin7	Finger millet	Overexpression of EcDehydrin7	[80]
Ec-apx1	Finger millet	Expression increased under drought	[82]
Mt1D	bacteria	Finger millet expressing mt1D had better osmotic adjustment and chlorophyll retention under drought	[81]
Metallothionein,	Finger millet	Induced under drought	[76]
Farnesylated protein ATFP6	Finger millet	Induced under drought	[76]
Farnesyl pyrophosphate synthase	Finger millet	Induced under drought	[76]
Protein phosphatase 2A	Finger millet	Induced under drought	[76]
RISBZ4	Finger millet	Induced under drought	[76]
β-carbonic anhydrase (PgCA)	Pearl millet	Up-regulated when exposed to drought	[79]

Table 5. Differentially regulated drought-related genes in millets.

Traits associated with drought tolerance were investigated using a genome scan and association mapping methods [77, 78]. A single gene known as β-carbonic anhydrase (PgCA) was

consistently up-regulated in pearl millet exposed to multiple abiotic stresses including drought, salinity and heat [79]. Hence, this particular gene is useful in adapting the plant to diverse abiotic stresses. Other genes known to be involved in drought response or tolerance in millets were EcDehydrin 7 [80], mt1D [81] and Ec-apx1 [82] from finger millet, and SiARDP [83] from wild foxtail millet.

Although not yet reported for millets, the suppression of two genes, namely, SAL1 and ERA1, increased the drought tolerance of the model plant *Arabidopsisthaliana* [84, 85]. The *era1* mutants develop tolerance to drought through a mechanism involving closing of the stomata [85].

## 5. Breeding millets for extreme drought tolerance

### 5.1. Germplasm acquisition and utilization

National and international efforts have been made to collect and maintain landraces of various millets types. The recent review by Goron and Naizanda [6] indicates the institutions involved in the preservation efforts and the amount of germplasm available at each institution. In general, India and China dominate the collections of millets. While institutions in India maintain 67% of the total of 33650 finger millet accessions, a single institution in China called the Chinese National Gene Bank preserves 61.2% of the total of 43,580 foxtail collections. Similarly, in the Ethiopian Institute of Biodiversity (EIB), over 5000 tef landraces collected from various tef-growing regions in the country are available [86]. Although these germplasm collections might not be exhaustive, they can play a key role in improving the productivity of respective crops. Further, large-scale expeditions need to be made for other millets in order to fully survey and bank the existing diversity in millets.

### 5.2. Breeding for drought tolerance

Breeding for drought tolerance is the major objective of many crop-breeding programmes due to the widespread prevalence of the moisture-deficit problem in global agriculture. A number of crops with drought tolerance have been developed. There are two options for the management of crops in water-limiting environments: the genetic and agronomic [87]. The genetic approach requires robust and reproducible screening methods for the identification of traits of drought tolerance in germplasm and breeding materials, and incorporation of the same into high-yielding varieties using conventional and biotechnological tools.

Crop breeding has relied for many years on conventional and ancient techniques such as selection and hybridization. Mutation breeding, the process of using chemicals or radiation to generate mutant plants with desirable traits, has also been used for several decades and has been a key in the release of over 2000 crop varieties to the farming community among which drought-tolerant cultivars are included [88]. Crop improvement techniques that apply modern genetic and omics (genomics, transcriptomics, proteomics and metabolomics) tools include the following: (i) *marker-assisted selection* (MAS) which refers to the utilization of molecular markers located near genes of interest to breed for traits that are difficult to observe, (ii) *TILLING* (targeting induced local lesions in genomes) [89] or *EcoTILLING* [90], the high-

throughput and non-transgenic techniques which rapidly detect point mutations in mutagenized populations, and (iii) *Gene* targeting that relies on the following three tools to increase the efficiency of gene targeting: *zinc-finger nucleases* [91, 92], *TALEN* (transcription activator-like effector nuclease) [93] and *CRISPR/Cas* (clustered regularly interspaced short palindromic repeats)/(CRISPR associated), type II prokaryotic adaptive immune system [94, 95].

### 5.3. Improved crop management

The wise use of crop management practices which include the time of planting, frequency of tillage and the rate and time of fertilizer application is important particularly in the semi-arid regions where moisture is scarce. Flexibility to change from late maturing crops to early maturing crops when the rainfall arrives late in the season is important. In the central semi-arid regions of Ethiopia farmers start their season by planting sorghum in April. When sorghum fails due to late arrival of rain, they sow wheat in June. However, if the rain is still late or not enough for wheat plant establishment, farmers sow tef in July or early August as the last option. Compared to sorghum and wheat, tef requires less moisture and matures early.

Suggestions have been earlier given on the type of technologies to be adopted in the semi-arid regions of Southern Africa [96] and West Africa [97]. According to Mir and colleagues, these technologies should include genomics, physiology and breeding [98].

### 5.4. Agricultural inputs and insurance

Access to agricultural inputs such as improved seeds, fertilizer and chemicals as well as credit and markets is important for farmers. In semi-arid areas where millets are dominantly cultivated, the amount and pattern of rainfall is erratic. Due to this, an insurance system known as Weather Index Drought Insurance has been implemented for the last decade in several African countries including Niger [99], Ghana [100], Kenya [101] and Burkina Faso [102] as well as India [103]. The successful insurance organization called 'Kilimo Salama' which was initially established by Syngenta Foundation for Sustainable Agriculture (SFSA) and implemented in several East African countries has been recently transferred to the Agriculture and Climate Risk Enterprise Ltd. (ACRE) [104, 105].

### 5.5. Partnership in research and development

Collaborations among national and international institutions are required in both research and development, in order first to develop improved millet cultivars and later to disseminate them to the farming community. Among the institutions with a global mandate to improve millets, ICRISAT (International Crops Research Institute for Semi-Arid Tropics) has recently added tef to the list of its mandate crops [106]. With its headquarters in Patancheru, India and regional officers in Nairobi (Kenya) and Bamako (Mali), it has been focusing on the improvement of diverse millets. The centre is among the 15 international agricultural research centers that belong to the CGIAR (Consultative Group for International Agricultural Research), the global partnership that unites organizations engaged in research for food security. Hence, the research and development of tef, a vital crop in the Horn of Africa that feeds over 50 million people in Ethiopia alone, will receive a global partnership towards its improvement and

development. In general, suggestions given to the improvement of understudied or orphan crops [107, 108] could also be applied to the research and development of millets.

## 6. Conclusions

Millets play a significant role in the livelihood of the population of developing world especially due to their enormous contribution to the food security of these countries. However, these crops have not been sufficiently studied and hence have been named orphan crops. Both conventional and modern improvement techniques have not yet been adequately implemented. It is believed that the changing climate will have significant effects on the types of crops cultivated in the next century. Currently, widely cultivated crops that provide the daily diet for many (such as wheat) might not be extensively cultivated in the future due to environmental stresses, especially the increase in global temperature. Millets might provide alternative climate-smart crops, as their adaptations to challenging environment are better than the current major crops of the world. Enhancing the productivity of millets requires concerted efforts of breeders, agronomists, policy makers and donors at both individual and institutional capacities.

## 7. Abbreviations

ABA; Absciscic acid

ACRE; Agriculture and Climate Risk Enterprise Ltd

AP or APX; Ascorbate peroxidase

CAT; Catalase

CGIAR; Consultative Group for International Agricultural Research

CSA; Central Statistical Agency (Ethiopia)

Ec-*apx1*; Ascorbate peroxidase

EIB; Ethiopian Institute of Biodiversity

FAO; Food and Agriculture Organization of the United Nations

FAOSTAT; Food and Agriculture Organization Statistics

GR; Glutathione reductase

ICRISAT; International Crops Research Institute for Semi-Arid Tropics

LEA; Late embryogenesis abundant

MDAR; Monodehydro-ascorbate reductase

Mt1D; Mannitol dehydrogenase

PgCA;  $\beta$ -carbonic anhydrase

POD; Peroxidase

PVC; Polyvinylchloride

ROS; Reactive oxygen species

SFSA; Syngenta Foundation for Sustainable Agriculture

SiARDP; *Setaria italica* ABA-responsive DREB-binding protein

SiLEA14; *Setaria italica* late embryogenesis abundant 14

SOD; Superoxide dismutase

UN; United Nations

WRSI; Water Requirement Satisfaction Index.

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# Plant Integrity – The Important Factor of Adaptability to Stress Conditions

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Additional information is available at the end of the chapter

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## Abstract

Crop production, research of crop productivity, tolerance to abiotic and biotic stresses, plant disease, and pests all represent the problem of plant integrity. Plants represent an integrated system of units, which are responsible for its resistance to adverse environmental conditions on the basis of the evaluation of characteristics both aboveground and in the roots. This "complete unit" (root and shoot) has an influence on the formation of seeds, the quality of which may affect subsequent growth, development, and stress tolerance of the filial generation. Properties of the roots predominantly influence (especially at drought stress conditions) growth, development, and the metabolic processes in the aboveground part of the plant. The seed traits affect the filial generation root morphology at the beginning of the vegetation period (especially length, surface, depth of root penetration, and also root weight). In the biology of the seeds, roots, yield formation, stress tolerance, etc., attention needs to be paid to plant integrity and adaptability during variable environmental conditions. Every plant, and its traits, is a result of all the plant's activities. This is important for plant breeding. For example, it is possible to provide selection for cultivar traits at seed germination. Quality of the embryonic traits is important for subsequent growth and development. In the juvenile phase, and in later stages, the same genotype is still active. This is among the main reasons for studying plant integrity.

**Keywords:** Plant integrity, abiotic stress, crop production, weather variability, seeds, roots

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## 1. Introduction

The effect of plant integrity and of aboveground/belowground defense signaling on plant resistance to abiotic and biotic stresses is a basic and very important subject of contemporary scientific research.

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Profitable, stable crop varieties demonstrate superior traits for all types of observed characteristics in all environmental conditions (seeds, roots, shoots). Good varieties in ecologically different conditions usually have superior features for all types of analyzed traits. It is possible to state on the basis of whole plant metabolism, that plants create integrated system units, which are responsible for resistance to adverse environmental conditions on the basis of evaluation of characteristics of their aboveground parts and their roots. This "unit" (root and shoot) has an influence on the formation of seeds, which, by their quality, may affect the subsequent growth and development of the filial generation, and thus is also resistant to external conditions.

The earliest information about "physiological manifestations of plant integrity" was found in ancient Rome, in the era of Gaius Julius Caesar. Plant integrity, regarding the "functionally coordinated whole plant body", was first defined on the basis of scientific knowledge in the Czech Republic by Rudolf Dostál (1885–1973). Properties of the roots significantly affect (especially during drought conditions) growth, development in the aboveground part of the plant, and plant seed growth and development. From the opposite viewpoint, it is known that seed traits affect, in the filial generation, root morphology at the beginning of the vegetation period: especially length, surface, depth of root penetration, and also root weight.

The first description of seeds was presented by the Greek scholar Theophrastus (372–282 B.C.). The main development of seed science began in the 19th century [1]. Seed traits are factors, with different levels of importance, which are given by soil and weather conditions during the sowing and time of emergence. The processes and traits of germination and field emergence of a plant are very important for subsequent growth and development. Genotypes with good seed germination under unfavorable conditions develop, in the filial generation, larger root systems in field conditions. However, the most physiological experiments with green parts of plants do not take into account the fact that "half" of the metabolic processes in plants take place in the roots. This part of the plant has an important influence on the shoot traits and seed production.

Darwin expressed that "roots are as brain of plants", i.e. roots can be taken as a similar body to the brain. Currently, it is known that for transmission signals (changes of potential) between roots and the aboveground parts of the plant, plasmodesma are needed. The root system provides transmission information to other parts of the plant (shoots and seeds under development). Information about pathogen attack or physical stress can be transmitted to the other parts of the plant in order to begin as soon as possible the organisms defense – thanks again to plasmodesma. If the stress is repeated again, the reaction of the plant is more rapid, based upon "plant memory".

In the biology of the seed, especially in seed productions we need to pay attention to plant integrity and adaptability over variable environmental conditions. Adaptability (plasticity) is a feature that allows the plant to return to its original previous physiological state, i.e. to basic metabolic functions, after unusual environmental conditions. Adaptability is fixed on all chromosomes and can be identified in the early stages of growth. Adaptability in a broader sense includes seed quality, especially the quality of sprouting plants, root system adaptability, and issues relating postharvest physiological processes, including the physiological processes



during storage. On the basis of entire plant physiology, it is possible to use more than 100 plant characteristics to improve adaptability under variable environmental conditions.



**Figure 1.** Sampling the root biomass for the evaluation of the relation between above and below ground parts of a plant.



**Figure 2.** Plant roots in the interior of Kateřinská Cave (Moravian Karst, Czech Republic).

## 2. Main abiotic plant stresses in central Europe

Abiotic stress is a main cause of reduced yield in the case of healthy plants. In this context, many scientific research projects have dealt with the impact of weather and climate change on agricultural crops. They were primarily focused on the impact of stress evaluation – especially drought and air and soil temperature extremes. The main current problems are lack of soil water or soil drought as well as high air temperatures. It has also been proved that there has been a prolongation of the growing season – 15 to 25 days – in central Europe in the last 20 years. This is accompanied also by an increasing probability of risk of vegetation frost. Concrete adaptation measures that would eliminate the impacts of climate change are still not a reality.

Monitoring of meteorological elements is crucial for the precise description of microclimatic conditions and their influence on plant physiological processes. The outcomes of microclimate monitoring provide valuable data for growth, plant protection, yield, and irrigation models as well as a wide range of other applications. Monitoring of air temperature and humidity as well as temperature and soil moisture and solar radiation should be an integral part of all growing trials – pot or field based. It is difficult to find any kind of cultivation experiment where the soil moisture and air temperature do not play important roles.

Globally, agriculture accounts for 80–90% of the freshwater used by humans. In many crop production systems such a water use is unsustainable. An interdisciplinary approach involving agronomical opportunities and plant breeding in order to deliver “more crop per drop” is needed [2]. In the field, the upper limit of water productivity for well-managed, disease-free, water-limited cereal crops is typically  $20 \text{ kg ha}^{-1} \text{ mm}^{-1}$  (grain yield per water used). Climate development in Europe since 1990 has been unfavorable for cereal yields because of heat stress during grain filling and drought during stem elongation. Drought during the generative phase decreases the number of based spikelets and grains. Another critical period is also flowering, when water shortage impact is worse than during other stages of development. During the stage of seed filling water stress disrupts the process of synthesis and storage of starch and storage proteins. It has been confirmed [3] that early drought reduces the number of offshoots and number of grains per ear. Late drought at the time of the development of leaves and grain filling causes leaves to age and their photosynthetically active surface decreases faster than in irrigated plants. Late drought negatively affects grain size.

Spring cereal yields decreased by  $45\text{--}75 \text{ kg ha}^{-1}$  due to decreased precipitation of 10 mm [4]. The highest values of water requirement in plants were observed in the stages from shooting to heading, during an intensive increase of biomass. During this period, the plants utilized up to 5 mm of water per day. Seasonal deficits of precipitation during the growing season in central Poland were -145 and -169 mm for barley and wheat, respectively. In the growing season they utilized from 293 to 314 mm of the soil water [5].

Moisture certainty analyses in the Czech Republic (central Europe) proved there was an increase in the driest areas and that drought event probability increased in during the 1961–2010 period [6]. An increase in air temperature above normal months and the loss of normal precipitation months were identified. An increase in temperature and precipitation extremes

in the future, across climatic conditions and types of landscapes in the Czech Republic, was found [7]. The occurrence of meteorological drought, as well as the occurrence of hydrological, agronomic, physiological, socio-economic, and other kinds of drought is an important feature of the Czech climate. Lack of soil moisture is expected in the main growing season (approximately 200 days) when the rainfall does not exceed 340 mm. In connection with the stress effects on yield and quality the most important indication for growers is the presence of agronomic drought. This is defined as a state where the amount of moisture in the soil is less than that required by a particular plant. Literature often defines agronomic drought as a decline in soil moisture below the permanent wilting point (i.e., approximately -1.5 MPa) which stops water uptake and, subsequently, plants growing. It has been claimed [8] that the proportion of usable water – not reducing yields – varies according to crop type and stage of development between 45% and 75% of the available water holding capacity (AWHC). [9] use soil moisture in the root zone at 65% AWHC as a limiting value for barley before transpiration is reduced.

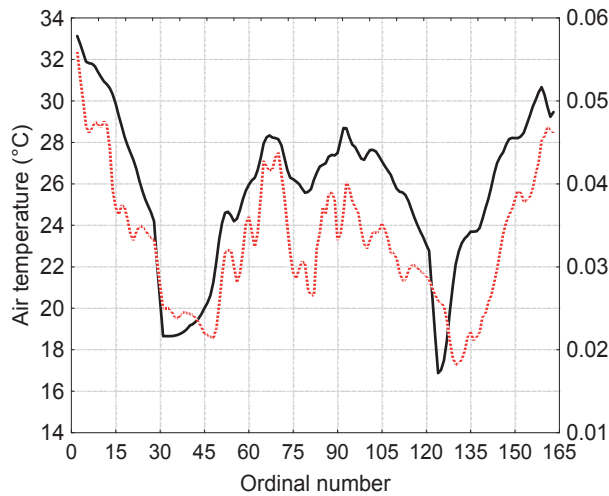
The availability of soil water, together with global radiation belong to the main agrometeorological elements which determine the transpiration performance of plants. Global radiation has a primary effect on the transpiration of plants, however, in the case of drought stress occurrence, one may expect a major influence to be played by soil moisture on the course of transpiration. A crop's reaction to a decrease in soil water capacity is different for different crop species. The high evapotranspiration requirements of the environment may cause a loss of soil water through excessive transpiration in non-sensitive plants.

Water shortage-induced stress often goes hand in hand with temperature stress. Transpiration is the main mechanism a plant has to protect itself against overheating. Leaf temperature increases with increase in air temperature. Effective use of water implies maximal soil moisture capture for transpiration, which also involves reduced non-stomatal transpiration and minimal water loss by soil evapotranspiration.

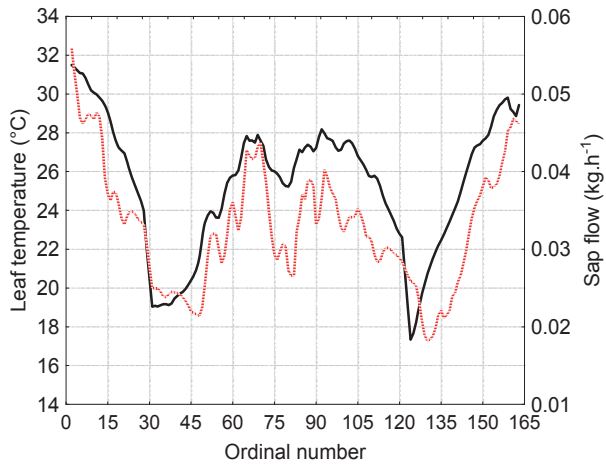
The dependence of maize transpiration on air temperature, air humidity, solar radiation, soil moisture, wind speed, and leaf temperature were quantified [10]. Significant relationships between transpiration, global radiation, and air temperature were found. Conclusive dependence of transpiration on leaf surface temperature and wind speed was found (Fig. 3–6). Transpiration in maize plants was significantly influenced by soil moisture under moderate and severe drought stress. The dependence of transpiration on meteorological elements decreased with increasing deficiency of water. A correlation between transpiration and plant dry matter weight, plant height, and weight of corn cob was found. These results will be utilized in an effort to make the calculations of evapotranspiration in computing models more accurate.

Breeding for maximal soil moisture capture for transpiration is therefore the most important target for yield improvement under drought stress. Conclusions have been made [11] that differences in the effective use of water expressed as different yields under the same conditions can be partly attributed to different root system sizes (RSS) (probably due to deeper rooting) and can be improved by breeding. A value of 55% AWHC [12] has been suggested as a

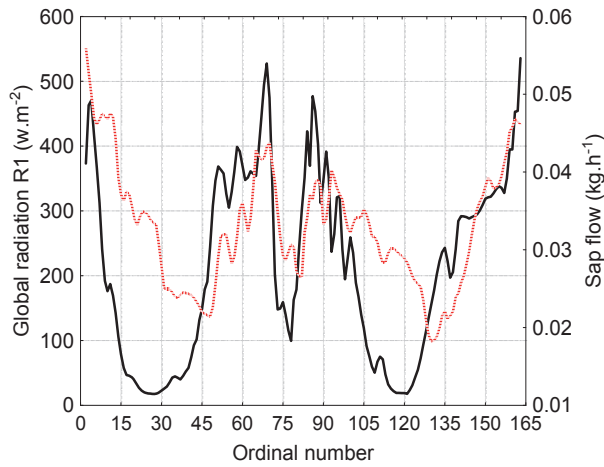
qualitative and not stressful value for all growth phases except at the beginning of flowering (45%) and plant maturation.



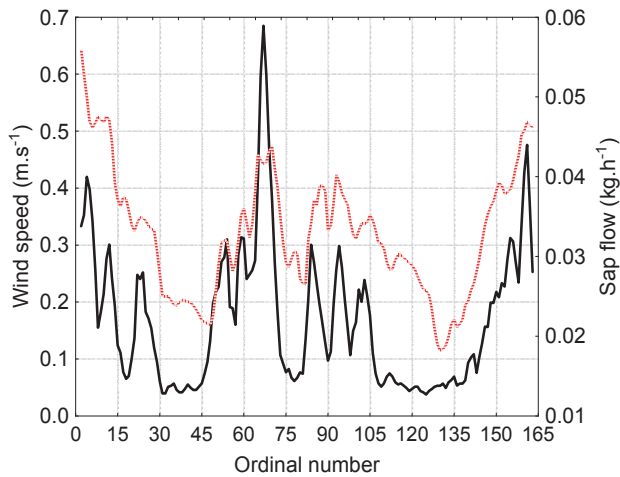
**Figure 3.** Course of sap flow (red line; kg h<sup>-1</sup>) and its dependence on air temperature change (black line; °C).



**Figure 4.** Course of sap flow (red line; kg h<sup>-1</sup>) and its dependence on leaf surface temperature changes (black line; °C).

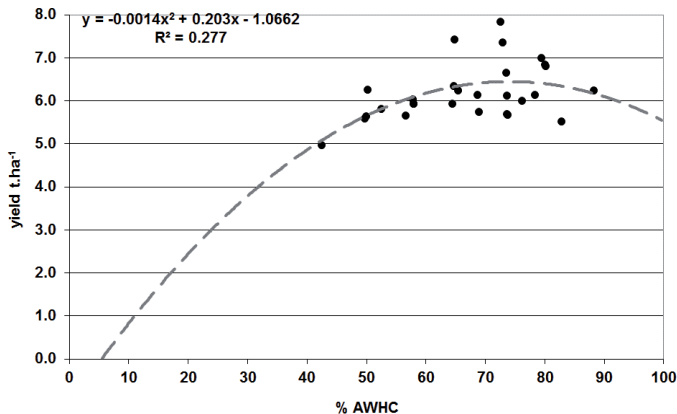


**Figure 5.** Course of sap flow (red line;  $\text{kg h}^{-1}$ ) its dependence on global solar radiation intensity (black line;  $\text{W m}^{-2}$ ).



**Figure 6.** Course of sap flow (red line;  $\text{kg h}^{-1}$ ) and its dependence on wind speed (black line;  $\text{m s}^{-1}$ ).

The amount of usable soil water was calculated using the agrometeorological model AVISO at 21 experimental sites for the period 1975–2007 (% AWHC) [13]. A decrease in usable soil water (% AWHC decrease up to 24%) in a growing season was observed at 20 localities in the long-term trend. Statistically significant relationships were found between grain yield of spring barley and level of AWHC (% AWHC). The optimum range for the amount of usable soil water for the production of spring barley (65%–75% AWHC) was defined by long-term calculations of soil water in combination with a series of yield trials (Fig. 7).



**Figure 7.** Relationship between the soil water supply (% AWHC) and yield of spring barley grain.

Decreasing winter precipitation, increasing winter air temperatures, and increasing levels of  $\text{CO}_2$  in atmosphere were forecast as global climate changes for central Europe. The negative effects of water stress were partially compensated for by elevated  $\text{CO}_2$  concentration. Warmer winters could lead to northward expansion of the areas suitable for cropping. However, for crops with a determinate growth habit (e.g. cereals) acceleration of development under warmer conditions could reduce the time available for growth before maturity thereby tending to reduce grain yield. Combining these effects with the fertilizing effect of increasing atmospheric  $\text{CO}_2$  concentration, yield of wheat could be 30%–55% higher if there is enough water [14]. For non-determinate crops (e.g. root crops) the warmer climate would extend the growing season. However, there is the possibility that the more frequent, damagingly high summer temperature events could reduce yields of both cereal and root crops. Water can be limiting not only due to global warming but also due to higher yields caused by new varieties and by higher levels of agronomic inputs. Breeding for greater RSS could be therefore one of the strategies for avoiding the impact of water stress. For example, the grain yield of winter wheat varieties in dry years is generally positively correlated with RSS. In a dry year, the varieties that showed the greatest difference in RSS were found to exhibit a yield difference of 860 kg  $\text{ha}^{-1}$ , approximately translating to an additional use of 15 mm of subsoil water [11].

### 3. Reasons why it is necessary to consider the integrity of plants in agriculture research

#### 3.1. Seed vigor

Seedling establishment is critical for production especially in stressed environments. The methods for the evaluation of seed germination are designed to have high levels of reproducibility and reliability; but worse than optimal conditions are often encountered in the field. For example, the germination percentage of barley (*Hordeum vulgare* L.) is an important character for both seed quality and malting quality. Although it is evaluated under optimal conditions for germination (laboratory temperatures and optimal moisture), it differs from germination in the soil and malting. In addition, seed lots that do not differ in germination may differ in emergence and storage potential. Seed vigor is generally described as the sum of the seed properties that determine the potential level of activity and performance of the seed during germination and seedling emergence [15]. Seed vigor is the ability of seeds to germinate and form the basis for future plant growth and development in standard and stressed conditions (drought, low temperatures, lack of nutrients). When soil conditions were unfavorable, the results of field emergence for wheat were more closely correlated with the direct stress vigor tests than laboratory germination [16]. The expression of seed vigor in field conditions and the translation to higher yields depends on the environment conditions.

Seed quality is of increasing importance as a result of climate development. Thus, seed vigor with regard to tolerance to drought and low temperatures as another potentially selectable trait which can be evaluated. The significance of this trait was documented by [17], who reported that an increase in the mean germination time due to poor seed vigor resulted in a significant loss in grain yield. The largest effect was found for winter wheat in which an increase in the mean germination time from 2.1 days to 3.6 days resulted in a relative loss in grain yield of 16%. It is possible to conclude, that increasing the sowing rates of low-vigor seed lots did not secure an optimal grain yield.

High seed quality may be particularly important in low-input agriculture because poor early performance is not as readily compensated for later on by mineral fertilisers and pesticides as it is in conventional agriculture. Furthermore, quality and seed vigor are important factors for competitiveness against weeds: the seeds of low vigor resulted in a perceptible increase in weed biomass and decrease in crop yield.

Various seed germination tests, under the suboptimal conditions of temperature, oxygenation, and water potential of the medium, or undergoing accelerated ageing and controlled deterioration allows for the sensitive differentiation between seed lots. Germination and vigor also depends on multiple biochemical and molecular variables, and its characterization is expected to provide new markers of seed quality that can be used in breeding programmes.

Significant correlations between field emergence and laboratory tests of vigor have been published [18, 19, 20]. Higher precipitation shortly before the harvest decreased the seed vigor of spring barley significantly [21, 22]. Higher air temperatures during this period and during the period April–July increased vigor significantly. Seed germination and vigor were related

to the parameters that are important for malting. The germination capacity of all lines was higher than their vigor and germination energy: 2.9% higher than vigor and 4.6% higher than the germination energy on average. This finding has confirmed the opinions of many authors who have reported that seed performance under optimal conditions is often higher in comparison to the seed performance in vigor experiments under stress and field conditions. Moreover, it has been confirmed that samples of the same germination capacity may have different vigor and storage potential.

The influence of late-terminal drought stress during grain filling on the germination and vigor of barley seeds has been studied [23]. Stress during the grain-filling stage had no effect on the standard germination test, but it obviously decreased the vigor of the seeds. The results indicate the positive influence of high air temperature during ripening and negative influence of high precipitation on the seed vigor.

The seed vigor of soybean, as evaluated as the mean percentage accelerated ageing rate, can be improved by breeding, whereas high yields were maintained because of the predominance of the general combined effects of both the seed vigor and yield [24]. The seed vigor, as evaluated by the cold test, showed estimates of the genetic response to selection in flax [25]. The three key traits of seed vigor in *Brassica oleracea* were rapid germination, rapid initial downward growth of the seedling, and a high potential for upward shoot growth in the soil with increasing impedance. This result suggests a strategy of stress avoidance. In addition, quantitative trait loci (i.e. QTL) were identified for marker and candidate gene identification. A few genomic regions (QTL) were identified for seedling vigor in rice. For these QTLs, significant genotype and environmental temperature interactions were found [26].

Our previous results [21] indicate the possibility of successful selection for higher seed vigor as an important factor of agronomic and malting quality, even in good years (vigor 93–95%), for the traits given above. However, in the years with generally much lower vigor (61–86%), the success could be more responsive because the effect of the variety prevailed over the effect of the environment for bad years. The vigor of 12 combinations from two locations was compared with vigor of their parents. Significant correlation was found between the vigor of the mothers and their progenies ( $r = 0.832$ ; significant on  $P \leq 0.01$ ), between that of the fathers and their progenies ( $r = 0.882$ ; significant on  $P \leq 0.01$ ), and between the vigor of both parents and their progenies ( $r = 0.894$ ; significant on  $P \leq 0.01$ ). This is further evidence for potential effective breeding for vigor.

A lower seed vigor was correlated with a high occurrence of fungi (as indicated by ergosterol assays) and to a lower percentage of field emergence [27]. Vigor was also related to bread quality [28]. Grain samples with 80–90% vigor produced the greatest bread volume. Grain with vigor below or above this range produced less voluminous loafs of bread. High-quality varieties had a higher content of total polyphenols than did varieties of lower quality and the polyphenol content was correlated to vigor.

Cultivars of wheat with enhanced early vigor are still not commercially available.



### 3.2. Plant roots

Contemporary knowledge confirms the possibility of selection for the root system and stress root tolerance on the basis of seedling stress tolerance, i.e. at time of the sprouting. It is possible also to evaluate characteristics of seeds and seedlings, i.e. provide selection, after plant hybridization of the plants on the basis of the seed and seedling traits, for seed quality an also classic selection in plant breeding.

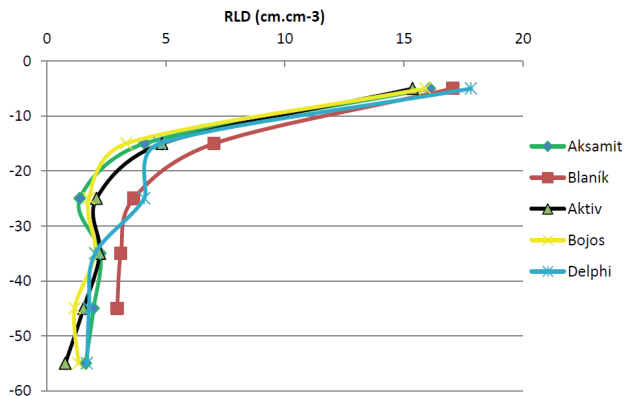
Plants have developed different root system size (=RSS) during evolution and breeding. In dry and low levels of nutrients in the soil environments a greater RSS is found enabling plants to be more efficient with their use of water and nutrients from lower soil layers. Varieties of cereals with greater RSS better use soil water and nutrients in dry environments than varieties with smaller RSS. Relations between RSS and yield level, variation, and quality should be studied in a broader range of environments in central Europe for agricultural crops. However, yield is a polygenic trait and its level cannot be therefore explained by variation in only one factor (e.g. RSS). The level is limited primarily by the factor in minimum as described by Justus von Liebig. During vegetation, further limits occur either at different or similar times.

The use of natural resources of agricultural and forest ecosystems is much dependent on the functioning of plant roots. These provide several goods and services to society in the forms of, e.g., yield production, carbon sequestration, avoidance of nutrient release from the soil, alleviation of floods, and energy production. The functioning of plant roots is much less well known than that of the shoots, mostly due to methodological reasons. Only special new technology allows us to address the whole root systems quantitatively. Roots are exposed to several stresses (e.g. water stress by drought, soil frost, hypoxia, water shortage by competition) during their lifetime which may decline their capability to provide goods and services. This is especially the case for trees whose lifespan ranges from tens to hundreds of years. Soil conditions will change with climate warming in many locations, linked to a change in precipitation in summer and winter seasons. Therefore, knowledge of the limits for stress tolerance of the roots of herbaceous and woody plants is demanded for future projections.

The “Green Revolution” created dwarf varieties capable of responding to higher fertilizer inputs without lodging, but failed to reach resource-poor farmers. Crossing early green-revolution wheat, with an  $F_2$  of Norin 10 or Brevor, reduced root biomass. Later generation, semi-dwarf wheat showed genetic variation for root biomass, but some generations exhibited a further reduction in root size [29]. Beside a better use of available water resources, an improvement in the uptake efficiency of nutrients from mineral and organic fertilizers would have an important economic and ecological impact for a resource-efficient agricultural strategy. Varieties with greater roots could enable better use of available nutrients and water, as shown e.g. for phosphorus. The selected varieties with greater RSS should be better adapted to soil problems, like lowering of groundwater tables, acidification, loss of organic matter, soil compaction by heavy machines, etc. Varieties with greater RSS could be bred as catch crops or for the phytoremediation of nutrients and heavy metals. Wheat genotypes with superior root characteristics for efficient nutrient uptake, especially during the tillering and booting stages, should be developed in breeding programs to increase grain yield and minimize nitrate leaching [30].

The effect of water and nutrient application on yield has led to the overuse of these practices in the last decades. This misuse of irrigation and fertilizers is no longer sustainable, given the economic and environmental costs. Transpiration stream largely determines the availability of the mineral N in the rhizosphere. This makes our poor estimate of root densities a major obstacle to any precise assessment of nitrogen availability in fertilized crops. A larger investment by the crop in fine roots at depth in the soil, and less proliferation of roots in surface layers, would improve yields by accessing extra resources. The economic return on investment in roots for water capture was twice the investment for nitrogen capture. An early and more extensive horizontal growth of wheat roots in the 0.2–0.7-m layer of the soil profile in glass-houses was found to improve substantially the uptake of N by vigorous lines [31]. There has been a long-standing interest in varietal differences in the uptake of nutrients, especially of N and P, but progress has been slow in translating this into information that can be used in breeding. Root systems limit plant breeding [32].

Breeding for RSS as a strategy for improving yield stability and crop productivity under dry conditions however is still largely ignored in the breeding process, when it is not the breeding aim as such, e.g. for root crops like sugar beet. The main reason for this shortcoming in breeding for drought tolerance is the lack of a suitable method for evaluation of RSS. An improvement in water use is relevant when soil water remains available at maturity or when deep-rooted genotypes access water in the soil profile that is normally unavailable. At moderate drought, productivity of cereals can be improved by a more effective use of available water, i.e. by increasing the plants access to a higher soil volume by a deeper root system and eventually an increased rooting density in deeper soil. Varieties with a deep root system (Fig. 8) should have more opened stomata to cool the plants by transpiration, and therefore improve their tolerance to high temperatures [33].



**Figure 8.** Vertical distribution of root length density (RLD) in spring barley varieties within the soil profile (Hrubčice, Czech Republic, 2012).

Better use of nutrients, including water, due to greater RSS, means:

1. lower the contamination of the environment by them, i.e. lowering the content of nitrates, phosphates, etc. in groundwater, rivers, and seas;
2. a higher adaptability to some stresses during plant growth and development, e.g. limited water, heat, and nutrients;
3. avoidance of water shortage caused by a lowering of the water table in the soil;
4. a better recovery after damage by the limited stresses and after disease and pest attack;
5. higher yields when other growing conditions are not limiting. However, when growing conditions are limiting and water and nutrients are not available, a greater RSS could be a disadvantage;
6. suitability for ecological and/or low-input farming; and
7. lower costs for crop production.

Serious environmental impacts were associated with an enrichment of surface water and groundwater by nitrogen and phosphorus. Increased intensity of livestock rearing depends in Europe in particular on large amounts of imports of nutrients – rich feedstuff from countries outside Europe. In specific areas this has led to nutrient surpluses, which have contributed to problems such as eutrophication.

There are a number of works that assess interspecies or intervarena differences in the above-ground biomass production of crops. Only a small number of authors deal with quantitative and qualitative assessment of underground biomass in relation to the dynamics of nitrogen in soil. An appropriate measure for the use of nitrate nitrogen from the soil in the autumn is the inclusion of cover crops into crop rotation. Field trials [34, 35] were executed to evaluate RSS in eight varieties of white mustard and five varieties of *Phacelia* on two locations, in three BBCH phases (i.e. international scale used to identify the phenological development stages of a plant) over two years. The relationship between RSS, aboveground biomass, and content of nitrogen in the soil was investigated. *Phacelia* featured on average a higher root/shoot ratio (0.45) than mustard (0.32), whereas the year impacted the ratio more than the production area type. In *Phacelia* a highly significant positive correlation was found between aboveground biomass and the amount of soil nitrate nitrogen. This phenomenon confirms that greater biomass production does not mean there is a lower soil nitrate nitrogen. A relationship between the RSS of mustard and the content of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  ions in the soil after harvest was observed. RSS negatively correlated with the content of nitrate nitrogen in the soil, however, the correlation was statistically not significant. Evaluation across sites revealed a positive correlation between aboveground biomass and the amount of residual  $\text{NO}_3^-$  ions in the soil. On the other hand a significant negative correlation ( $r=-0.81$ ) of RSS and  $\text{NH}_4^+$  ions content was observed.

The effect of drought stress on the monitored traits of the root system and aboveground biomass of spring barley (*Hordeum vulgare* L.) was evaluated in a pot experiment by [36]. The characteristics of three varieties in a three-year observation in four different irrigation treatments were evaluated: length, surface area, weight of the root system (evaluated by the soil-core method with subsequent digital image analysis), the RSS (detected by measuring its

electrical capacity), and dry matter yield of aboveground biomass and root biomass – the shoot ratio. Dry matter yield of aboveground biomass significantly correlates with the RSS ( $r = 0.700$ ; significant on  $P \leq 0.01$ ). The variability of root system traits was affected by year (40%–50%), treatment (10%–11%), and variety (8%–14%). Weight ratio of aboveground biomass and root:shoot ratio were affected largely by variant (28.1% and 42.0%; significant effect). Year worked at least root: shoot (15.6%; significant effect). Variants without stress produced the most above ground and below ground biomass. However, the root:shoot ratio was the lowest in this case.

### 3.3. Examples of effective selection for greater root system

RSS was one of the selection criteria in the breeding for dinitrogen fixation. Breeding of varieties with greater RSS (alfalfa Zuzana), and greater RSS and higher dinitrogen fixation (alfalfa Nitro, white clover Nivel) has been successful [37].

In cereals, root densities of 1.0–1.5 cm cm<sup>-3</sup> are needed to extract plant available water from the soil, e.g. [38, 39]. Maize plants rarely achieve this below 70 cm, but values of 3–5 cm cm<sup>-3</sup> or more are common in the top 30 cm of soil. For better exploitation of available water, a better distribution of roots in the soil profile is preferable to partitioning more dry matter to roots [40]. A field trial with selected varieties of spring barley has been realized [36]. For five varieties RSS, its vertical distribution in the soil profile layers up to a 60-cm depth, and grain yield were evaluated. The impact of locality, year, and variety on root system attributes was quantified. Highest values of root length density (RLD) were determined in the layer between 0 cm and 10 cm (Figure 8). A tendency to increase RLD in both research localities and in most varieties in the layer between 40 cm and to 60 cm was detected. A significant dependency of grain yield on RLD was only determined in the middle layers of the soil profile. In wet years a significant negative correlation was determined.

The RSS has been found to be a genetic trait, and some specific genes have been observed to control this property. In wild barley, the gene *Hsdr4*, involved in water-stress tolerance, was located on chromosome 3H near *sdw1*. This was identified as a marker of QTL for great RSS [41]. Therefore, the RSS and drought tolerance per se maybe linked.

The isolation of intact living root systems from soil in the field has not yet been published and seems impossible. This difficulty is evidenced in many observations. Biomass estimates from minirhizotrons indicate that the <0.25 mm diameter roots (Fig. 9) account for nearly 95% of the total root length [42]. Root separation using a sieve with a 0.5-mm mesh screen led to a marked underestimation of root length density and root biomass. Values up to three times higher were observed when using a 0.2-mm mesh screen in comparison to a 0.5-mm screen [43]. Ex situ methods are expensive and connected with relatively high experimental error. More progressive are in situ methods, in particular use of electric capacitance. Comparison of this method with the ex situ soil-core method found a good correlation. The electric capacitance can be therefore recommended as a quick and cheap method, which enables repeated evaluation of vegetation and retains the evaluated plants until harvest.

The capability “to see” the roots in the soil plays a key role in the evaluation of the potential of herbaceous and woody plants to produce goods and services for society and in the detection of the stress thresholds of roots. From a functional point of view, fine roots (Fig. 9) are the most important for plants. Thus, a method that gives a measure of the root surface area for absorbing water and nutrients would be valuable and it would offer wide applications for users of the natural resources of plant/forest ecosystems.



**Figure 9.** Measurement of the finest structures of a root system is possible using the method of electrical capacitance.

Wheat (*Triticum aestivum* L.) has been systematically bred for about 200 years. However, this breeding has been done using only aboveground plant parts. We evaluated previously the roots of 18 wheat populations [44]. The RSS was evaluated by its electrical capacitance directly in the field (in situ). The RSS of plants in third and fourth generations were evaluated during shooting and heading. In these evaluations plants were selected for large and small root systems. In dry environments, the progeny of plants with large and small root system had yields of 17.1 and 10.9 grams per plant in the third generation and 18.5 and 10.0 grams per plant in the fourth generation (Fig. 10). The selection process showed a greater response for larger root system size. Selection for higher wheat RSS can be easily used to breed for drought tolerance and higher efficiency of water and fertilizer use.

The study [45] introduces the evaluation of RSS for the breeding of spring barley, in particular for drought tolerance. The aim of this study was to present the method of RSS evaluation and show it in practical use, in particular in relation to drought tolerance. The varieties of spring barley were evaluated for RSS by its electrical capacity. The RSS was compared with the grain yield and grain quality of the varieties at 7–19 stations each year. Varieties with a greater RSS had a significantly higher yield in the dry part of the year. Varieties with a greater RSS had significantly higher contents of starch, saccharide extracts, and malt extracts, as well as higher

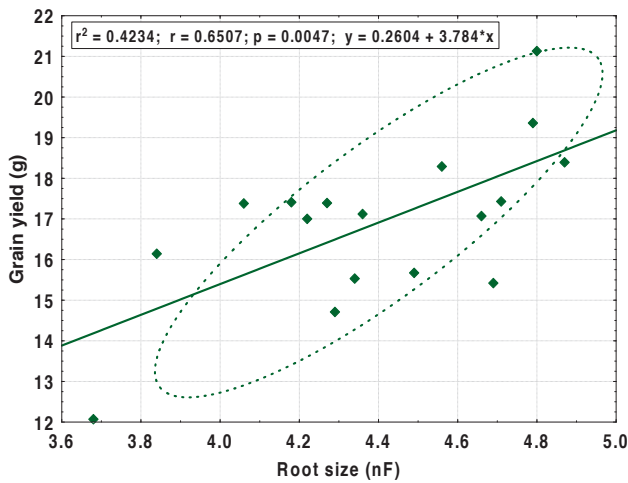


Figure 10. Regression relationship of the wheat grain yield on RSS as averaged from three locations (published in [44]).

yields of protein and starch in dry environments. It can be concluded that a small RSS is related to a low grain yield and malt quality in dry environments, even in genetically diverse varieties.



Figure 11. The root is the most sensitive organ of the plant. On the left: tree sample *Pinus silvestris* (Scots pine), standing on the main root, on the 4 m height, due to influence of strong soil erosion. These trees have on the basis of measurements at least twice as large a root system compared to those in a conventional environment. In the other two images are oil rape roots. The left of these images is a root located from a relatively dry, well-prepared soil. The right of these images shows a root from compacted, moist soil. Such changes have an effect on the metabolism of plants, yield, seed quality, stress resistance, i.e. not only variety, provenance, and method of seed storage. In addition, the quality of soil preparation (at field crops) has a large influence.

The relevance and response to selection for greater RSS of spring barley in field conditions has been studied [46]. The effect of selection in 12 barley populations developed by mutual crossing



of 4 parents in the  $F_3$  generation as a response to the selection in the preceding generation  $F_2$ , for great and small RSS was evaluated. The selection was effective. The average difference in the parental segregating generation was +40 and -43%, and in progeny +4 and -2%, respectively. Grain yield responded two times more to the selection (plants with a root size greater by 3.9% had higher yield of 8.1%). Root size was found to be related to grain yield. Some varieties donated greater, some smaller roots into progeny. It was shown that the selection for RSS was effective and responsive (more for greater than for smaller roots) in a similar way as for grain yield and can be therefore used in practical breeding.

#### 4. Importance of plant integrity in crop research

Plant integrity looks like as a “very easy and expanded topic,” but the reality is very different. “Thanks” to the rigid specialization of scientists, we are losing a holistic view of plants. It is necessary to sense a plant in its entire complexity – both roots and shoots, as well as across their life cycles. Complex research, i.e. the connection of biology, plant nutrition, ecology, and other disciplines, is hardly observable in most scientific work. Only such an integrated approach can allow us to reach the correct interpretations for experimental results [47–54]. For example, when interaction of three or more stressors exists, there can be a lower or higher effect of stressors (compensated through the course of metabolism, etc.). The reason is the influence of individual factors on metabolism and their possible antagonistic influences. Stressors always have pleiotropic effects on a plant, and influence on many genes. Therefore, it is very important to test the adaptability of new breeding materials by utilizing extreme conditions in more locations, such as in ICARDA (The International Center for Agriculture Research in the Dry Areas) and CIMMYT (International Maize and Wheat Improvement Center) in Mexico [54, 55].

Currently, research is booming for the use of model, standard plants (*Arabidopsis* etc.). It is desirable that agricultural research is conducted with crops and not with so-called model species. The reasons for this requirement is both practical and physiological (diversity of metabolism). If, for example, *Arabidopsis* is used as a “model plant” for oilseed rape, because it is (on the basis of a physiological viewpoint) a similar plant with regards to its metabolic functions, then why not start research work straight away with oilseed rape?

There is also other problem. The differences between biological and agricultural research has increased in many cases. This phenomenon can also be observed in the relationship between contemporary agricultural research and its practical applications. Narrow research specialization predominates strongly, and the differences between biological and agricultural research has increased in many research areas. There is a relatively significant lack of new methods for the analysis of this problem. To be honest, lot of different types of internal plant signals, i.e., the transport of information in plant metabolism and their interconnection, memory of plants, and importance of plant neurobiology is also neglected [56].

Productive genotypes have a more efficient metabolic activity throughout all vegetation, with faster spring root growth and better plant cover affecting their subsequent growth and

development. In our experiments, the influence of provenance was greater than that of variety (this is not a rule, it may be the opposite due to differences between varieties and locations at each experiment). Provenance has a very significant influence on the physiological characteristics of seeds, an even greater influence than seed weight and germination. The results confirm the importance of quality seeds to the cultivation of field crops and especially highlights the importance of plant integrity [57–62]. For example, analysis of the effect of variety and provenance of oilseed rape seeds (*Brassica napus* L. ssp. *oleifera*) on germination under different temperature conditions was provided. The obtained results confirm the importance of seed provenance and emphasize a preference for high-quality seeds. It is known that good seed vigor and provenance guarantees not only better field emergence, growth, and development of overall resistance to stressors (drought, high temperature), but also has a significant impact on seed yield in the subsequent generation (according to better overwintering).

There are several ways to improve the resistance and tolerance (adaptability) of plants to the variable environmental stress conditions with regard to the basis of the whole plant physiology.

The last physiological studies on the rape plant integrity and plasticity have shown, that the crop “responds” to stressors by more than 100 of currently known and measured physiological traits (as mentioned in the previous section of this chapter). There is possibility to use this phenomenon in plant breeding. Selected basic traits of seeds as a vigor, germination percentage, emergence, and stress tolerance of germinated seeds to high and low temperatures has significant influence on the yield of winter oilseed rape varieties [57–63], see below. These problems require more detailed analysis (64–67). Adaptable varieties have a wider possibility for growth and development in field conditions. Unfortunately, the current system of approving new varieties in many European countries is not heading in the direction of adaptable varieties, but rather in the direction of morphological homogeneity of the genetic material.

4.1. The results of experiments with the oil rapeseeds

Genotype	Country of origin	Company origin
Californium	France	Monsanto SAS
Viking	Germany	NPZ (Norddeutsche Pflanzenzucht)
Navajo	Great Britain	CPB Twyford Ltd
OP 4947/07	Czech Republic	Oseva PRO, s.r.o.
Cadeli	France	Monsanto SAS
Grizzly	France	Société RAGT 2n
ČŽL 20	China	Breeding material
Labrador	France	SCA Adrien Momont et Fils

Table 1. Oil rapeseed cultivars at additional tests



On the basis of laboratory additional tests with the selected cultivars (Tab. 1) the following results supporting the importance of the integrity of plants were obtained:

1. It is possible to eliminate, at the seed level, genotypes (initial breeding materials and cultivars) which do not tolerate extreme temperature and temperature changes during germination, have low water use efficiency, and are intolerant to abiotic stresses.
2. It was confirmed that these genotypes also have poor field emergence and initial growth of roots with implications for a further vegetation period, mainly for overwintering and spring regeneration which has influence on the yield.
3. This test method represents a tool for the screening of genetic resources with resistance to abiotic stress and this technology process is acceptable also for other crops.

Presented results were confirmed in the other rapeseed varieties (36 cultivars). Important obtained relationships are given in Tab. 2.

Seed germination after exposure to cold temperatures for three days after the saturation of seeds by water – simulating the impact of early frosts (20°C/12 hr a day, and -3°C/12 hr a night)	+0.56**
Seed germination after exposure to high temperatures for three days after the saturation of seeds by water – simulating the impact of high temperature (30°C/12 hr a day, and 20°C/12 hr a night)	+0.47*
Influence of the locality (provenance)	+0.78**
Influence of the cultivar	+0.68**
Efficiency of water utilization (water content required for germination) at standard conditions during sprouting	+0.35*
Efficiency of water utilization at high-temperature conditions during sprouting	+0.88**
Efficiency of water utilization at low-temperature conditions during sprouting	+0.25

Note: Stress tolerant seeds at time of sprouting are one of the lot of guarantors of the of quality crops establishment during growth and development and for winter survival.

\*\*Statistical significance at 1%; \*Statistical significance at 5%

**Table 2.** Correlation coefficients: seed physiological traits and the yield.

However, one very important problem in plant experiments remains. There is the possibility to select pairs of cultivars with different traits and for simplification from the same latitude it is possible to obtain many conclusions. In this case there is a random drift of results. If we use large groups of cultivars it is possible to obtain, for different physiological types, common and repeatable conclusions.

The cultivars used for scientific experiments are not often appropriate for the analysed target. Shortly: There is in lot of “physiological experiments” with comparison of only two contrasting

genotypes (varieties). It is very little number for finding general dependencies. (Then there plays the role random drift as mentioned). This type of scientific work has both physiological and genetic aspects, logical reasoning during the writing of scientific articles, and is at a high level; we can read a productive scientific article, but the practical application of the results for plant production and breeding then becomes impossible. It is not possible to draw general conclusions based on the analysis of two genotypes especially if whole plant physiology is important to the solution of the problem.

## 5. Conclusions

It is possible to provide selection for cultivar resistance to stress already at the seed germination stage and on the quality of plant root systems. Quality of the embryonic roots is important for the following growth and root development. Why does this possibility exists? The answer is because in the juvenile phase and in later stages, the same genotype is still active.

The effect of plant integrity and of aboveground–belowground defense signaling on plant resistance against abiotic stresses (and also pathogens) is important and a relatively new subject of scientific research. Biochemical analysis has unequivocally proven that plant defense responses to stress and pathogen infection differ between whole intact plants and detached plant parts (leaves, roots, stems, etc.). Therefore, the question arises – which type of scientific work will reflect reality more realistically? It is possible to conclude that it is research on the basis of plant integrity.

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# Plant Evolution in Response to Abiotic and Biotic Stressors at “Rear-edge” Range Boundaries

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Additional information is available at the end of the chapter

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## Abstract

Most species in the mustard family are restricted to higher elevations and latitudes where they also have restricted local spatial distributions. In this chapter, we describe a novel hypothesis for the development of low-elevation range limits in upland mustard species. The hypothesis suggests that defense regulation of glucosinolates could underlie the evolution of the spatially restricted distributions. A list of testable predictions is presented to evaluate the hypothesis. An interdisciplinary Ecological Genomics approach is needed to test the predictions; therefore, we also describe the field of Ecological Genomics. Although there is already support for some of the predictions, which we discuss, most of the predictions remain untested. Therefore, we also describe several tests that help evaluate each of the predictions.

**Keywords:** Range limits, signaling pathways, crosstalk, evolutionary constraints, ecological genomics

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## 1. Introduction

Mustard plants (Brassicaceae) include approximately 3,700 species, several crop species (cabbage, radish, canola, etc.), and the model for molecular plant biology, *Arabidopsis thaliana*. Despite this diversity, mustard species generally inhabit high-altitude temperate regions where populations have patchy distributions (Al-Shehbaz personal communication). At lower elevations and latitudes, species often face both abiotic and biotic stressors, which populations must adapt to for range expansion or to survive climate shifts. Mustard species are also characterized by the production of glucosinolate defense toxins [1]. In this chapter, we evaluate a recent hypothesis [2] that regulation of glucosinolates could underlie the evolution of the

spatially restricted distributions. This hypothesis is in contrast to previous hypotheses on defense evolution that argue the opposite that variation in defensive chemistry is the consequence of spatial distributions, life history patterns, inherent growth rates, etc. [3, 4]. As such, we begin this chapter with a description of the central hypothesis, followed by a set of predictions and then a description of the interdisciplinary approach needed to evaluate the hypothesis.

## 2. Hypothesis

Understanding the causes and dynamics of naturally occurring range limits in plants has become a central issue in both basic (evolutionary ecology) and applied (conservation and agriculture) areas of biology because of climate change and land use concerns [5-8]. Most transplant studies show decreased performance just across geographic range boundaries ([6] for review); therefore, it is generally assumed that many range boundaries are spatial manifestations of niche limits, requiring adaptation for local range expansion or for the persistence of populations at range edges as climate changes. However, the existence of range limits suggests that adaptation to stressful environments just outside the range is often prevented. What prevents this adaptation from occurring?

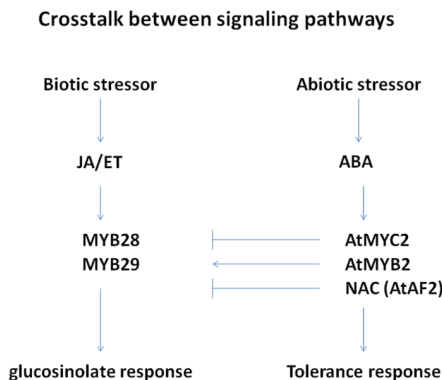
Many factors and processes can contribute to range limit development. These factors include lack of genetic variation in range margin populations, barriers to dispersal, gene flow from elsewhere within the range, and various kinds of tradeoffs [6]. Any of these factors, alone or in combination with other factors, could prevent adaptation to stressful environments outside the range. However, there is often sufficient genetic variation within and among range margin populations [9] for natural selection to presumably act upon, and often there are no obvious barriers to dispersal at range boundaries. In these cases, possible constraints on the process of adaptation to stressful environments just outside the range would include gene flow and tradeoffs. But because many range margin populations are geographically and genetically isolated (e.g., [10]), it is thought that the study of range limit development should often focus on molecular, physiological, or developmental tradeoffs [11]. What kind of tradeoffs might be important to mustards at low elevation or low latitudinal range limits?

The process of adaptation often proceeds by modifying existing structures and pathways. Within ranges, stress response signal transduction pathways help plants to survive temporary challenges from abiotic and biotic stressors [12]. Just across range boundaries, some of these same stressors increase in frequency; therefore, one would predict that adaptation to stressful environments across range limits would involve the upregulation of stress response pathways such that the pathways and the traits that they regulate were expressed more frequently or stably.

However, evolutionary models predict that a problem may arise when antagonistic response pathways are co-opted simultaneously for evolutionary change [13]. The problem is with negative pleiotropic and epistatic effects. Multiple signaling pathways often form networks of regulatory genes (transcription factors) that may interact for multiple positive and negative integrative effects. An excellent example is the flowering time signaling network in Arabi-

dopsis, which involves many positive and negative interactions among photoperiod, circadian clock, vernalization, autonomous and Gibberellic acid pathways. In general, quantitative geneticists predict that the evolution of complex traits may involve many genetic correlations, which is why there has been such an interest in the analysis of quantitative genetic variance–covariance G-matrices [14]. Indeed, early theoreticians such as Fisher predicted that the evolution of complex traits would only involve many genes of small effects to avoid such pleiotropic effects [15]. Epistatic interactions between major flowering time network genes *FRI* and *FLC*, for example, were one of the contributing factors in the maintenance of genetic variation in *Arabidopsis* flowering time [16] and therefore may represent an evolutionary constraint. *FRI* and *FLC* are major transcription factors (TFs) in the flowering time signaling network that allow large behavioral shifts involving many genes, but these major effects might impede evolution through multiple epistatic and pleiotropic effects.

In another example, it is generally assumed that plants face both abiotic and biotic stressors across range boundaries, especially at low elevation “trailing edges” of species ranges [17], yet it is well known that stress response pathways, such as abscisic acid (ABA) signaling for coping with abiotic stressors (e.g., drought) and jasmonic acid (JA) signaling for coping with biotic stressors (e.g., herbivores) may negatively interfere with one another ([18 - 20] for reviews). Thus, the simultaneous co-option of these antagonistic pathways for low-elevation range expansion where organisms face both increased abiotic and biotic stressors may be problematic because of the crosstalk. Although limited phylogenetic evidence suggests that ancient antagonistic crosstalk between signaling pathways may not constrain evolution [21], more thorough and experimental work is needed to address this issue.



**Figure 1.** Crosstalk between JA/ET and ABA signaling. MYBs, MYCs, and NAC are transcription factors. The key candidate TFs in *Arabidopsis thaliana* either regulate aliphatic GS toxins (MYB28 [At5g61420] and MYB29 [At5g07690]) (44-48) or regulate both defense and drought tolerance pathways (AtMYC2 At1g32640, AtMYB2 At2g47190, and a NAC TF AtAF2 At5g08790) (18, 19, 20 for reviews).

Specifically, our hypothesis states that components of defense (e.g., JA – jasmonic acid) and stress tolerance (e.g., ABA – abscisic acid) signaling pathways are not genetically independent of one another, which may constrain the simultaneous evolution of defense and stress

tolerance. Candidate genes in *Arabidopsis* for the evolutionary constraint include specific TFs that are involved in gene regulation within and among signaling pathways (Figure 1). For example, if there exists genetic variation in TFs MYC2 or AF2 that natural or artificial selection could act upon to increase drought stress tolerance traits for more stable expression, we predict that [1] a glucosinolate defense response would also change because these TFs help to regulate both pathways, and [2] other pleiotropic or epistatic effects would reduce fitness.

### 3. Predictions

1. Defense and stress tolerance phenotypes will be genetically correlated in family structured common garden experiments.
2. Genetically diverged populations from different elevations will also be diverged for defense and abiotic stress tolerance traits; neither population will have high values of both traits simultaneously.
3. Defense allocation and stress tolerance phenotypes will not segregate independently from one another in extended generation crosses between diverged populations.
4. Defense and stress tolerance genetic covariation will be associated with markers linked to candidate transcription factors (TFs) that regulate both defense and drought tolerance pathways (AtMYC2 [At1g32640], AtMYB2 [At2g47190], and a NAC TF AtAF2 [At5g08790]).
5. DNA sequence variation of candidate regulatory genes (TFs) implicated in prediction #3 will also correlate with the defense and stress tolerance tradeoff in (a) the segregating crosses and in (b) unrelated individuals (linkage disequilibrium association analysis). Comparison of DNA sequence of coding regions will also show molecular evidence for evolution (McDonald-Kreitman Test and dS/dN ratios).
6. There may be other regions of the genome besides those containing the candidate TFs that also simultaneously affect defense and stress tolerance phenotypes and thus would also provide molecular evidence for the tradeoff. For whole genome marker analysis away from candidate TFs, defense and stress tolerance phenotypes will either co-locate in linkage mapping or their QTL will show negative epistasis.
7. Gene expression of candidate genes in genetically diverged populations will reflect the evolution of more stable expression of defense and stress tolerance response pathways.

### 4. Approach and Discussion

An Ecological Genomics approach would satisfy the need for experimental and molecular genetics to evaluate the hypothesis in several of the predictions. Ecological Genomics (EG) is an interdisciplinary approach in the biological sciences that seeks to find the genes underlying

species interactions in natural habitats and to study the evolutionary forces that have shaped these genes, their expression patterns, and the phenotypes that they encode [22-26]. As such, nucleic acid sequencing, forward and reverse genetic tools, comparative methods, and other molecular techniques are required to find the relevant genes, to establish databases of candidate genes, and to study their expression patterns and allelic variants.

Although many model species (species whose genomes have been completely sequenced and that have broad interest from molecular biologists) meet and exceed these molecular criteria, often they lack the attributes for ecological studies. For example, the natural distribution of *A. thaliana* is in Eurasia, making it difficult for study by North American researchers. Furthermore, there are still relatively few model species. With the advent of affordable high-throughput next-generation sequencing (NGS), however, sequence information is becoming available for more species and populations. As of June 2015, for example, there were 6,653 completely sequenced genomes, but only a small fraction of these are eukaryotes (<http://www.genomeonline.org>). The GOLD website also listed 60,631 genome sequencing projects (June, 2015), but only 9,059 or 15% were eukaryotes. Thus, although NGS is helping the field of EG to move away from model species, especially, for example, in evolutionary studies [27], there is still a need to understand the attributes that constitute an ideal model organism for EG studies.

Feder and Mitchell-Olds [22] listed the criteria for an ideal model species in Evolutionary and Ecological Functional Genomics (EEFG), which is synonymous with EG. The criteria stated that there needs to be: (1) a co-operative community of researchers from different disciplines that share resources and information; (2) the tools to find genes and study their variation within and among species; (3) natural, undisturbed habitats such that genetically diverged populations can be studied for local adaptation; (4) molecular data on sequences and chromosomal maps for marker development and mapping, cis and trans regulatory regions identified, and gene function and its fitness consequences known under natural conditions; and, finally, (5) the ability to study the ecological consequences of natural genetic sequence variation in the genes for evolutionary inferences. Thus, access to NGS alone does not necessarily make a species ideally suited for EG study.

*Boechera stricta*, a close wild relative of *Arabidopsis*, satisfies many of these criteria and is an emerging ecological model species that inhabits environments that differ substantially in drought stress, herbivore community, and other abiotic and biotic conditions [28]. The selfing rate of *B. stricta* in the northern portion of its geographic range is 0.95 [29], enabling the creation of experimental advanced generation hybrids for forward ecological genetic studies (e.g., [30]). The genome of *B. stricta* has also been recently sequenced ([http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org\\_Bstricta](http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Bstricta)).

Forward genetics for finding candidate genes in ecology include population genomics, association mapping, linkage mapping, and transcriptomics [31, 32]. Population genomics identifies outlier marker loci in the statistical analysis of population genetic parameters, but there is no knowledge of associated phenotypes. Association and linkage mapping both include measurement of phenotypes, but a distinct advantage of association mapping is that no pedigree is required and that allelic variants representative of naturally occurring populations can be used in analyses [26, 33]. Association mapping can be conducted on unrelated

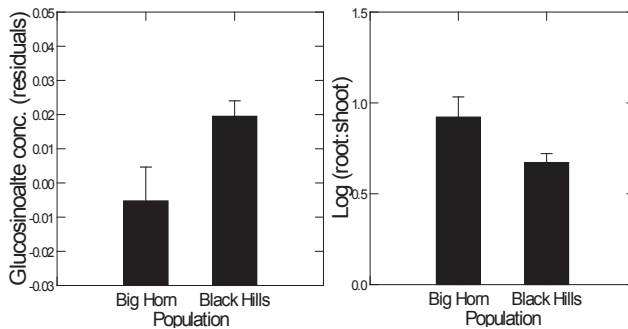
individuals because it is based on general inherent linkage disequilibrium (LD). When markers for association analysis are developed from candidate genes, significant associations may actually identify relevant genes. We assume that candidate genes in *Arabidopsis* could often be studied with success under natural conditions in close relatives in the genus *Boechera*. Unidentified causal genes in LD with significant candidate markers are unlikely in *B. stricta* where LD decays rapidly, within 10 kb [34]. If present, population substructure (i.e., stratification, admixture, or inbreeding) must be controlled for genetic association analyses because of confounding effects on LD that can lead to false positives [33].

#### 4.1. Tests of prediction #1

Using family structured quantitative genetic analyses, previous studies have examined the genetic correlation between defense and stress tolerance of prediction #1. This work has been conducted on a close wild relative of *Arabidopsis* in the genus *Boechera*. *Boechera stricta* is a genetically diverse, diploid, predominantly self-fertilizing species that occurs at higher elevations throughout western North America in natural habitats [29, 34]. The phenotype of an individual of any species is determined by genetic and environmental factors ( $P = G + E$ ), and these factors and thus the phenotypes vary among individuals within populations ( $V_p = V_G + V_E$ ). If the phenotypes are measured from in a common garden experiment, environmental variation is eliminated so that the phenotypic and genetic variations are equal ( $V_p = V_G$ ). Among full-siblings of clonal or self-fertilizing species such as *B. stricta*, the genetic variation ( $V_G$ ) measured from a common environment can be used for evolutionary inferences because all allele combinations within and among loci are inherited without change. In accordance with prediction #1, the negative genetic correlation between glucosinolate (GS) toxin defense allocation and stress tolerance associated with range limits of *B. stricta* has been observed using these methods in the field and lab five times previously [2, 35-37]. Stressors involved in the tradeoff have included drought, nutrient deficiency, and change in plant community structure across the range boundary (suggesting competition) and the multivariate stress of the range boundary itself. In these studies, it was hypothesized that these tradeoffs occurred because of antagonistic crosstalk between abscisic acid (ABA) stress tolerance and jasmonic acid/ethylene (JA/ET) or salicylic acid (SA) defense-signaling pathways. Circumstantial evidence implicating the pathways in the tradeoff comes from experimental ABA soil inoculations that depended on endogenous GS level [35]. These conclusions are based mainly on correlative methods and circumstantial evidence. Experimental and molecular genetics and more direct measures of pathway components are needed.

#### 4.2. Tests of prediction #2

For gene mapping in *B. stricta*, a replicated cross has been conducted between populations from the Big Horn Mountains, Wyoming, and the Black Hills, South Dakota. These are geographically isolated and genetically diverged populations [36, 38]. The populations are located at different ends of the altitudinal range of *B. stricta* (Big Horns 3,000 m, Black Hills 1,700m) and thus the sites differ by several environmental factors. The populations have diverged for glucosinolate content and stress tolerance traits such as root:shoot ratio as predicted; neither population had high values of both traits (Figure 2).



**Figure 2.** Genetic variation in GS levels ( $F_{18, 701} = 7.101$ ,  $P < 0.001$ ) (left) and root:shoot ratios ( $F_{3, 251} = 3.576$ ,  $P = 0.004$ ) (right) between genetically diverged populations. Data are from a growth chamber experiment. Residuals controlled for plant size, thus GS levels reflect content.

### 4.3. Tests of prediction #3

The segregating F2 populations from the crosses can be used to test prediction #3 that the defense and stress tolerance traits will not be inherited independently of one another. F2s from more than one cross allow for broader inference and increased statistical power [39]. Because each genotype in the F2 segregating generation cannot be replicated, drought treatments in the lab would need to be imposed on all plants after the plants were first monitored for performance (e.g., growth) under controlled watering conditions. Measures of drought tolerance could include relative growth (before and after drought treatments), leaf mass area (LMA), water use efficiency (carbon isotope ratio), and root:shoot ratio. Several measures of stress tolerance increase the probability of detecting the tradeoff, and provide a general assessment of stress tolerance [40]. Glucosinolate analysis should be conducted on leaf tissue at the time of drought stress.

Of course, the goal should be to test for the tradeoff in the field across the low-elevation range boundary using the segregating generation of the experimental crosses. However, this requires replication of each F2 genotype that could be compared within and just outside the range boundary. In the F3 generation, there is replication of each F2 lineage. Common garden experiments of F3 families could be established within and across low-elevation range boundaries. Previous field transplant experiments [2, 35, 36] have established the areas just across the low-elevation range boundary as stressful in terms of several correlated abiotic and biotic stressors, which manifest as slower growth and survivorship. Large sample sizes would guarantee the detection of effects if they exist, and would also allow for assessment across multiple years and the possibility of ecological gradient manipulations. For example, removal treatments of candidate competitors, such as *Lithophragma parviflorum* [36], could be included within and outside the range. Competitive interactions also induces ABA [36] and therefore might also induce the tradeoff.

#### 4.4. Tests of predictions #4 and #5

Here the goal is to test whether TFs can be implicated in the tradeoff using TF-linked markers in the crosses, and then to conduct further genetic association analyses using markers within the implicated candidate TFs. Previous molecular work allows one to locate markers on a *B. stricta* linkage map that are linked to the candidate TFs in the crosses. Chromosomal painting and end sequencing have shown that there are large syntenic blocks that align between the *A. thaliana* and *B. stricta* genomes [41]. These Arabidopsis blocks have been located in the *B. stricta* genome. This can now also be verified with the recently sequenced *B. stricta* genome.

Analyses within the candidate genes could be conducted in populations of related and unrelated individuals. The analyses of related individuals could use F2 and F3 mapping populations, but there would be confounding effects of linkage and linkage disequilibrium (LD) from other unknown genes and alleles. Genotyping in the F2 generation also allows for genetic association analyses in the F3 field experiments. Significant associations using unrelated individuals would help to eliminate confounding effects of linkage and linkage disequilibrium (LD) from other unknown genes and alleles. Another set of markers such as microsatellite markers (e.g., [29, 36]) would need to be used to identify and control population genetic structure among the unrelated individuals [33].

For marker development within genes of *B. stricta*, identification of genetic variation (e.g., single nucleotide polymorphisms – SNPs) in marker-implicated candidate TFs can be performed by first sequencing the TFs and their promoter regions from the diverged populations. Alignment of the diverged sequences would identify any polymorphisms. The recently sequenced *B. stricta* genome allows one to readily design primers to amplify and then sequence the genes.

To test whether polymorphic genes are under selection, statistical tests of synonymous to nonsynonymous substitution ratios in coding regions would be performed. Comparing dN/dS ratios allows for detection of positive or negative selection, albeit conservatively. The MacDonald-Kreitman test is more liberal for detecting deviation from the neutral model of molecular evolution [42].

#### 4.5. Test of prediction #6

To determine whether drought tolerance and defensive QTL co-localize on the *B. stricta* genome or whether there are epistatic interactions between QTL from different traits, linkage mapping would need to be conducted in F2 lab and F3 field experiments. Genotyping conducted in the F2 generation can also be used for successful mapping among F3 sib families [43]. Linkage analysis is often extended to incorporate information from several markers, called multi-point or interval mapping; therefore, composite interval mapping followed by multiple interval mapping using QTL-Cartographer will be used.

#### 4.6. Test of prediction #7

If natural selection has acted upon genetic variation in signaling pathways for more stable or frequent expression of traits, this should be detectable in a comparison of the high- and low-



elevation populations for trait responses and gene expression. For example, the populations could be compared in a double challenge experiment in which plants are first drought-stressed and then fed upon by generalist insect herbivores. One would predict that drought stress responses would take precedence over herbivore-induced defense responses in close wild relatives of *Arabidopsis*, reflecting the crosstalk between ABA and JA signaling that has been well documented in *Arabidopsis*. But we would predict that this plastic tradeoff would vary between high and low diverged populations and that this would be reflected in differential gene expression involving the candidate signaling pathways. To examine the expression levels of candidate TFs, one could use qPCR, but genome-wide gene expression using RNAseq would also allow the assessment of other genes as well.

## 5. Conclusion

An Ecological Genomics approach is needed to evaluate most of the listed predictions that remain untested. We mainly advocate a candidate gene approach that leverages the vast functional genomics literature of *Arabidopsis* for functional ecological genetics studies on close wild relatives. However, next-generation sequencing is rapidly increasing the potential of many ecological systems. The suggested studies outlined here are important because if our hypothesis continues to be supported, there may be important implications for understanding range limits, defense evolution, canalization, conservation, and crop improvement. If defense regulation can be used to help predict population sensitivity to environmental stress, then there would be several important applied implications.

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# The Trials and Tribulations of the Plant Male Gametophyte — Understanding Reproductive Stage Stress Tolerance

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Additional information is available at the end of the chapter

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## Abstract

Yield and productivity of many crop species depend on successful reproductive development to produce seeds or fruits for human nutrition. Plants determine the right time to flower based on environmental cues (day length, temperature) and angiosperms have evolved a plethora of mechanisms to adapt flowering to specific environmental conditions. Despite these adaptation mechanisms, fertilisation and seed production remain subject to the reigning weather conditions before and during flowering. To fertilise the immobile female gametes inside the ovule, the male gametophytes need to be dispersed in a hostile environment. In crop plants, unexpected inclement weather conditions during male gametophyte development and pollen dispersal are often associated with dramatic yield losses. Molecular and physiological studies are gradually making progress in identifying genes and processes that control various aspects of pollen development, but the many intricacies involved in environmental control of pollen development and – in particular – regulation of male fertility remain poorly understood. The aim of this paper is to draw attention to the enormous amount of complexity and biodiversity that exist in angiosperm male gametophyte development. A better understanding of the strategies that exist in adapting pollen production and fertility to environmental challenges may ultimately benefit improvement of abiotic stress tolerance in major food crops.

**Keywords:** Male gametophyte, pollen, development, abiotic stress, angiosperms, fertility

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## 1. Introduction

The reproductive cycle in plants alternates between a haploid gametophytic and a diploid sporophytic generation. During the evolution from green algae (Charophytes) to land plants, the dominance of the gametophytic generation has gradually decreased in favour of the

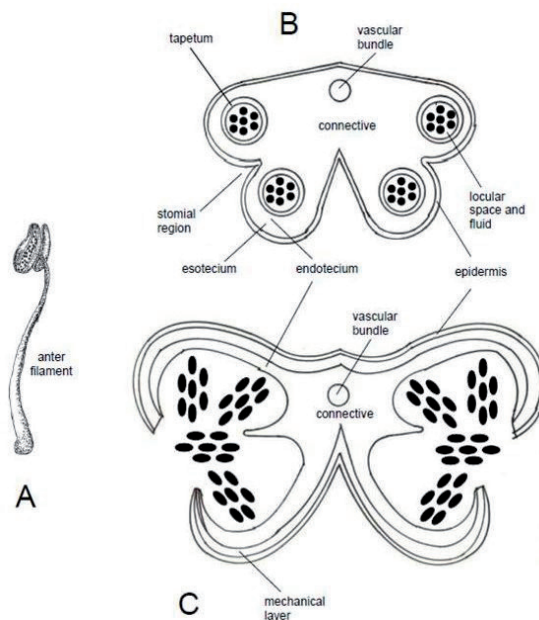
sporophytic generation. Originally, the gametophyte and sporophyte were separate independent organisms with very different appearances. In the first non-vascular land plants (liverworts, mosses) the gametophyte was still dominant, but in the first vascular land plants (ferns) the sporophyte prevailed. In ferns, the gametophyte is still an independent organism but with vastly reduced size. In seed-producing higher plants (Spermatophyta: angiosperms, gymnosperms), the gametophyte reduction became extreme (only a few cells) and both male and female gametophytes became physically part of the sporophyte [1, 2]. The emergence of the sporophyte as the dominant phase of the life cycle in seed plants has been attributed to genetic complementation and the capacity of the diploid stage to mask deleterious DNA mutations, an idea that was supported by the fact that land plants had to adapt to a more hostile environment. This argument has been disputed and the exact reason why the diploid sporophyte stage became dominant in land plants is still being debated [1, 3–5].

The ecological pressure to adapt to a dry environment with exposure to many new environmental stresses (water stress, UV light and heat) required a lot of morphological and developmental changes during the evolution from mosses and ferns (Archegoniatae) to Spermatophyta [6]. The generation of the vascular system, roots, stomata and the hormonal system that regulates these developmental features in Spermatophytes evolved along with adaptation to new environmental challenges [7–9]. The next step in the evolution of land plants was the establishment of sexual reproduction in a land environment and the development of gametophytes with different sizes and sexes (heterospory). Sexual reproduction offers an opportunity to recombine combinations of genetic traits and spread genetic variability between populations. This new-found capacity played a major role during evolution in the adaptation of plants to the terrestrial environment [10–12]. Sexual reproduction became therefore the prevalent reproduction system in both plants and animals [13]. The immobility of the sporophyte in land plants makes pollen and seeds the only vector systems to exchange genetic information between plant populations. Pollen production and pollination are critical in the breeding system of land plants, and the large biodiversity that evolved in plant pollination mechanisms illustrates the tight linkage with environmental adaptation [11, 14].

The origin of pollen can be traced back to heterosporous Pteridophyta (vascular plants) [15–17], which have microspores with features that are reminiscent of pollen: similar cell wall (intine and exine), storage reserves for the first stages of growth, reduction or absence of watery vacuoles at maturity [18]. In seed plants, the female gametophyte is immobile and develops totally inside the ovule of the ovary [19]. This makes pollen grains a crucial mobile vector for exchanging genetic information between different plant populations. The male gametophytes form inside the pollen sac in gymnosperms and in the anthers of angiosperm flowers (Figure 1) [2, 20, 21]. Pollen grains need to be dispersed from the anther and travel to the stigma to fertilise the immobile egg cell inside the ovule(s) of the ovary. This ovary can be located in the same flower, another flower of the same plant, a neighbouring plant or a more remote plant. The tough multi-layered pollen wall is an adaptation to protect the male gametes against environmental stresses during presentation and dispersal, while it is at the same time adapted for different pollen dispersal methods [22, 23]. The pollen dispersal methods and breeding systems in plants are amazingly diverse [14, 24]. Following domestication, many crop species



are grown in environments that are vastly different from their original growth habitat. The breeding system of many crop species may therefore not be optimal for their current growth habitat, let alone whether it will be adapted to a future world with a different climate. In many staple crops (e.g. rice and wheat) male reproductive development is considered the ‘Achilles tendon’ of reproductive development, with massive yield losses under unexpected adverse weather conditions (heat, drought, cold) becoming increasingly common occurrences [25–28]. Although the generation of haploid male gametes in angiosperms occurs via a conserved pathway, there are many variations present in different plant species in the way this process proceeds. In many cases, this biodiversity can be associated with adaptations to particular environmental restraints. This paper will explore the complexity in angiosperm pollen development and investigate how it can contribute to a better understanding of abiotic stress tolerance of male reproductive development. The focus of this review paper will be on the interaction between environment and pollen developmental processes and not on the diversity that exists in pollen–stigma compatibility and plant breeding systems. A supplementary glossary of commonly used terms and definitions related to male gametophyte development is supplied for those readers who are less familiar with this subject (See Appendix).



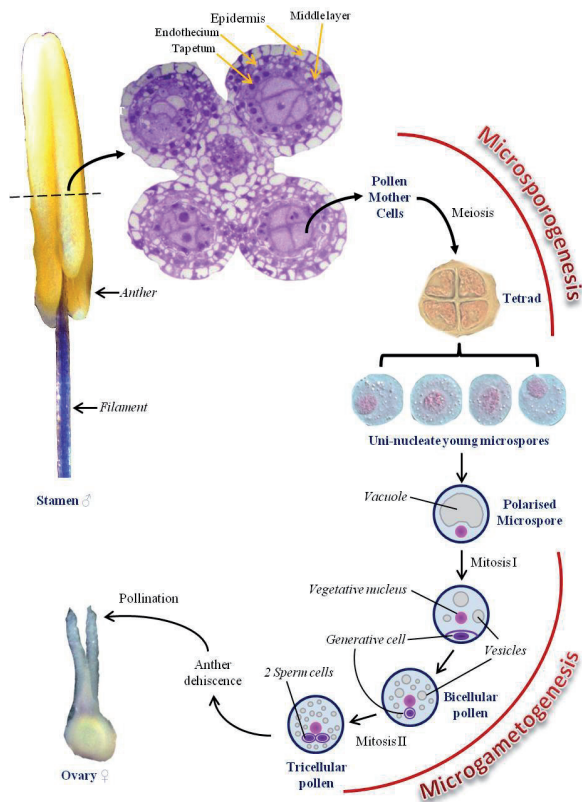
**Figure 1.** Schematic drawing of an ideal stamen (A), anther at microspore stage (B) and just after anther opening (C) with their components and functions. Water and some nutrients are transported by the vascular bundle from the mother plant via the filament towards the anther. Nutrients move to the tapetum via the connective tissue and components synthesised by the tapetum are then released into the loculus, where they are absorbed by the developing grains, and they are either utilised immediately or stored temporarily in the locular fluid, vacuole or amyloplasts.

2. Male gametophyte development and its biodiversity

In angiosperms, male gametophytes develop in the anther. Each anther consists of two thecae, each consisting of two adjacent microsporangia that are separated by the connective tissue (Figure 1). The first phase in pollen development, the meiotic division of the sporophytic meiocytes of the four microsporangia to form haploid tetrads and young microspores, is called microsporogenesis (Figure 2). During the second phase, microgametogenesis, the microspores enlarge and become vacuolated. Vacuolisation and the cytoskeleton force the nucleus to migrate to a peripheral position. The first mitotic division is asymmetric and produces a germ cell that is engulfed by the cytoplasm of the vegetative cell to become physically isolated from the vegetative cell (bi-cellular pollen; cell-within-a-cell). The germ cell then undergoes a second mitotic division to produce the two sperm cells (Figure 2). During fertilisation, one male gamete fuses with the egg cell and the other with the two polar nuclei of the central cell to form the zygote and endosperm, respectively. The male sperm cells are very diminutive in size, but transcriptome analysis has recently revealed that their gene expression pattern is unlike any other plant tissue, suggesting that they are functionally very specialised [29].

Pollen type	Starch content	Two-celled	Three-celled
Orthodox (>20% water)	Starchy	<ul style="list-style-type: none"> <li>· <i>Olea europaea</i> (Oleaceae) PK</li> <li>· <i>Erica arborea</i> (Ericaceae)</li> </ul>	<ul style="list-style-type: none"> <li>· <i>Wolfia arrhiza</i> (Araceae) (PK)</li> <li>· <i>Lilium bienne</i> (Liliaceae) (PK)</li> <li>· <i>Nelumbo nucifera</i> (Nelumbonaceae) (PK)</li> </ul>
		<ul style="list-style-type: none"> <li>· Solanaceae (PK presence depends on pollination syndrome)</li> <li>· Lamiaceae (PK)</li> <li>· Myrtaceae (PK)</li> </ul>	<ul style="list-style-type: none"> <li>· <i>Hedera helix</i> (Araliaceae) (PK)</li> <li>· <i>Borago officinalis</i> (Boraginaceae) (PK)</li> </ul>
	Starchless	<ul style="list-style-type: none"> <li>· Scrophulariaceae (PK)</li> <li>· <i>Acanthus mollis</i> (Acanthaceae) (PK)</li> <li>· <i>Bryonia dioica</i> (Cucurbitaceae) (PK)</li> <li>· <i>Cucumis melo</i> (Cucurbitaceae) (PK)</li> <li>· Liliaceae (some species) (PK)</li> </ul>	<ul style="list-style-type: none"> <li>· Caprifoliaceae (PK)</li> <li>· Asteraceae (PK)</li> <li>· <i>Canna indica</i> (Cannaceae) (PK)</li> <li>· <i>Tulipa gesneriana</i> (Liliaceae) (PK)</li> </ul>
		<ul style="list-style-type: none"> <li>· <i>Cucurbita pepo</i> (Cucurbitaceae) (PK)</li> </ul>	<ul style="list-style-type: none"> <li>· Amaranthaceae (PK)</li> </ul>
Recalcitrant (<20% water)	Starchy	<ul style="list-style-type: none"> <li>· <i>Plantago</i> sp. (PK)</li> <li>· <i>Portulaca tuberosa</i> (PK)</li> </ul>	<ul style="list-style-type: none"> <li>· Alismataceae (PK)</li> <li>· Poaceae</li> </ul>
	Starchless	<ul style="list-style-type: none"> <li>· <i>Laurus nobilis</i> PK</li> <li>· Malvaceae PK</li> </ul>	<ul style="list-style-type: none"> <li>· <i>Cereus</i> sp. (Cactaceae) (PK)</li> <li>· Caryophyllaceae (PK)</li> </ul>

**Table 1.** Classification of pollen diversity according to cytological events during pollen development, and examples of some representative plant species. Pollenkitt (PK) is typically present in zoophilous and entomophilous species and is generally absent in anemophilous species, with the exception of *Olea europaea*, a secondary anemophilous species. Some plant families (e.g. Liliaceae) have a majority of members with two-celled starchless grains and some species with three-celled starchless pollen grains [30, 185] (E. Pacini, personal observations).



**Figure 2. Male gametophyte development in angiosperms.** Pollen grains develop in the stamen, which consists of a filament supporting the anther. The vascular bundles in the filament conduct nutrients from the mother plant to the anther. The cross-section of the anther (rice) before the onset of meiosis shows the four microsporangia where the male gametophytes develop. From outside to inside, the anther wall consists of the epidermis, the endothecium, the middle layer and the tapetum. Both the middle layer and the tapetum degenerate towards pollen maturity, leaving only the epidermis and the endothecium to protect the pollen grains in the loculus before anther opening. The central cells of the anther, the pollen mother cells (meiocytes), differentiate and become selectively isolated from the mother plant through callose secretion by the meiocyte cytoplasm. The pollen mother cells undergo meiosis to form tetrads. The uni-nucleate young microspores are released from the tetrad with the help of enzymes secreted by the tapetum [313]. Exine is completed with the intervention of polymers secreted by the tapetum in the loculus [35]. Young microspores have a central nucleus and in Poaceae they are with the pore attached to tapetum until anther opening. The germination pore becomes visible and a large vacuole forms, pushing, with the intervention of the cytoskeleton, the nucleus in a peripheral position (polarised microspore stage) [176, 314]. At the vacuolated stage, the microspores undergo an asymmetric division (pollen mitosis I) to produce the vegetative and generative nucleus. The generative nucleus is then isolated in a separate compartment within the vegetative cell to form a bi-cellular pollen grain (cell-within-a-cell). During pollen maturation, the vacuole of the vegetative cell gradually decreases in size and accumulation of starch granules is observed (engorgement). In plants with tri-cellular pollen, a second mitotic division of the germ cell takes place before anthesis (pollen mitosis II) to produce the two sperm cells. At this stage, the two germ cells are found in close proximity of the vegetative nucleus (male germ unit).

Nuclei number (meiosis, tetrad, microspores), pollen grain cell number (bi-cellular and tri-cellular pollen) and other cytological events (vacuolisation, starch accumulation/hydrolysis, water content) are used to determine pollen developmental stages (Figure 2). These parameters can differ between plant species and differences in pollen development can be used for systematic classifications (Table 1). At dispersal, angiosperm pollen grains can be bi-cellular or tri-cellular (Table 2) [30]. In tri-cellular pollen, the second mitotic division occurs prior to dispersal and pollen is dispersed with the two sperm cells already formed (Figure 2). In bicellular pollen, the second mitotic division occurs during pollen tube growth inside the style-stigma. The term male germ unit describes the relative position and cytological connections between the generative cell, the sperm cells and the vegetative cell nucleus in the mature pollen and pollen tube [2, 31]. Very few species release bi- and tri-cellular pollen grains at the same time. When this occurs (e.g. *Annona cherimola*), the ratio between bi-cellular and tri-cellular pollen grains was shown to depend on environmental factors such as temperature regime and relative humidity during the last phases of maturation [32]. Tri-cellular pollen grains have completed their development before dispersal and are typical for plant families that include important dicot and monocot crop species such as Asteraceae, Lamiaceae, Brassicaceae and Poaceae (Table 1). In some plants, pollen is dispersed as aggregates containing a high number of pollen grains (e.g. massulate orchids) [33]. Orchids are monocots that produce bicellular pollen; the generative cell is spherical at dispersal but changes to the normal spindle shape prior to the second mitotic division when pollen lands on the stigma and starts emitting the pollen tube [34]. Pollen development is further subdivided in early, middle and late stages according to cytological and morphological features such as the presence of a vacuole (Table 1; Figure 2) [35–37]. Vacuolisation occurs only once in some species, but twice in others (once during the early microspore to bi-cellular stage and once during early bicellular to late microspore stage) [18]. Stages of pollen vacuolisation alternate with stages of starch accumulation in plastids (engorgement) and starch accumulation can therefore also occur once or twice. Mature pollen grains can be starchy or starch-less depending on whether starch is present in mature exposed pollen grains (Table 1). Another classification is based on water content of pollen at dispersal: orthodox and recalcitrant pollen is dispersed in partially desiccated or partially hydrated form, respectively. Other differences concern the presence or absence of pollenkit that distinguish animal/ insect from wind pollinators, respectively (with rare exceptions; Table 1). The diversity in pollen development between different plant species is complex and is functionally important. Different mechanisms have evolved under a variety of environmental constraints to secure pollination success and survival of the species.

### 3. Meiosis: The start of reproductive development

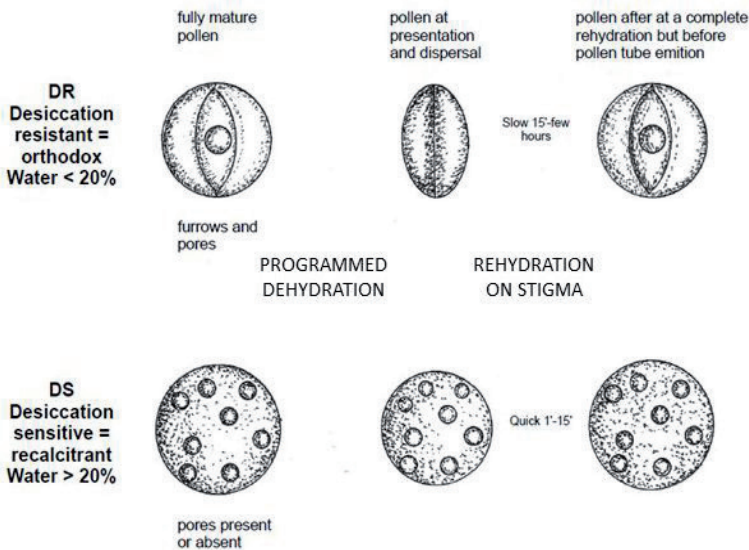
The decision to flower in higher plants is carefully controlled by environmental stimuli such as temperature and photoperiod [38–42]. After floral meristem initiation and formation of flower buds, meiosis is the committed step for sexual reproduction and formation of the gametophytes. The onset of meiosis is regulated by signals coming from the mother plant. Sugar availability plays an important role in driving cell division by inducing expression of

<i>Locular space availability</i>	<i>Pollen number</i>	<i>Pollen density and dispersal unit</i>
Abundant, space between pollen	Few pollen/locule	6–12 per loculus cross section (Poaceae)
	Many pollen/locule	15–30 per loculus cross section (Solanaceae, Fabaceae, Liliaceae)
Reduced, closely packed pollen	Septate anthers	Compound pollen in each septum, polyad type (8–32 pollen) (Mimosaceae, some Annonaceae)
	Aseptate anthers	Monad pollen, tightly packed, tetrahedral shape (Myrtaceae)  Compound pollen, very high pollen number, reduced size (Orchidaceae, Asclepiadaceae)

**Table 2.** Table showing the presence and abundance of locular space and fluid and relationship to pollen dispersal units in angiosperms. The locular fluid volume is extremely reduced when the pollen dispersal unit is of the compound type. Locular space and fluid are present from meiotic prophase until anther desiccation and opening.

the cell cycle regulatory protein cyclin that induces meiosis [43–46]. The initiation of meiosis to form the male and female gametophytes in the anther and ovary is normally a synchronised process [47–52]. However, this is not always the case in some plants and abiotic stresses can cause asynchrony between male and female meiosis [47, 51]. Most commonly, in aseptate anthers all sporogenous initials will proceed to undergo meiosis, while in septate anthers only some initials will undergo meiotic division [53]. This difference will affect locular space and liquid volume available to pollen, pollen number per locule and ultimately the dispersal unit (Table 2). After meiosis, male and female gametophytes follow a very different path of development. While ovule development and maturation is a gradual process, formation of large amounts of pollen grains in the anthers is energetically more demanding. At the time of meiosis, the anthers represent the highest sink strength in the flower and anthers are known to have the highest soluble sugar content of any plant tissue [54, 55]. Synchrony of male meiosis can also be affected in interspecific hybrids [50]. Pollen sterility caused by meiotic asynchrony is a major problem in interspecific rice hybrids where productivity is affected [56]. Mutagenesis approaches in model plants are gradually revealing genes that are involved in initiating meiosis and its progression through the different phases [57–61]. Silencing of the anther-specific zinc finger transcription factor *MEZ1* causes abnormal meiosis and pollen abortion in petunia [62]. The Arabidopsis *STUD*, *TAM*, *DUET*, *MALE MEIOCYTE DEATH1*, *AtKIN14a*, *b* and *TETRASPORE* genes are responsible for different aspects of male meiosis, such as maintaining pace, synchrony, chromosome organisation and transition between different stages [63–68].

Pollen biotechnology is a potentially powerful tool for crop breeding. Genes that regulate progression and synchrony of pollen meiosis and their regulation (e.g. effect of abiotic stresses) can be exploited for establishing hybrid breeding technologies, for instance, using mutant lines that are conditionally arrested at pollen meiosis [69, 70]. Progress in understanding pollen meiosis will be accelerated by more refined technologies that make it possible to study the meiotic transcriptome in detail [71]. Transcriptome profiling has been used to investigate the







**Figure 4.** Change of shape and size of pollen according to their water content during the more critical phases of dehydration, presentation, dispersal and rehydration. High temperature and low relative humidity affect desiccation-sensitive pollen (DS) more than desiccation-resistant pollen (DR).

effect of abiotic stresses on pollen meiosis and pollen development [72, 73]. Abiotic stresses such as cold during meiosis can lead to formation of diploid gametes [74]. Polyploidisation and manipulation of chromosome number during meiosis can be used to increase diversity in breeding of crop plants [75, 76]. Some *Arabidopsis* mutants (*DIF1*, *TETRASPORE*, *PARALLEL SPINDLE1* and *Jason*) that affect ploidy levels can improve our understanding of pollen meiosis and how it is affected by the environment [66, 77–80].

#### 4. The importance of the anther tapetum

The tapetum surrounds the pollen mother cells before meiosis and is the inner cell layer of the anther wall (Figure 2). The tapetum plays an important role in pollen development: it secretes the locular fluid containing nutrients for pollen development and deposits components of the pollen cell wall. When these functions are fulfilled, the tapetum undergoes a natural programmed cell death response (PCD) [81–83]. This process is essential to sustain pollen development: PCD generates nutrients for the locular fluid to feed the native pollen grains [81, 83–88]. Tapetum cells are generally polyploid and/or multinucleate and are metabolically very active. Tapetal-specific gene transcripts are the most prevalent fraction of total anther transcripts [89]. Polyploidisation and genome endoduplication are commonly observed in plant tissues with high metabolic activity [90]. High

<i>Pollen size</i>	<b>Water content</b> <b>Shape (dehydrated)</b> <b>Examples</b>	<i>Time for pollen rehydration and tube emission <sup>(1)</sup></i>	<i>Consequences</i>
<b>15–30 <math>\mu\text{m}</math></b>	[H <sub>2</sub> O] < 20% (Orthodox)  Boraginaceae, e.g. Forget-me-not	30 min to 1 hour	· Rehydration and pollen tube emission in extra-stigmatic sites of the flower or other plant parts may occur when air relative humidity rises
	[H <sub>2</sub> O] < 20% (Recalcitrant)  Spinach, Parietaria, Urtica	From a few seconds to a few minutes	· Time for rehydration and germination is less for recalcitrant pollen and depends on water percentage during stigma adhesion · Due to their small size they lose water quickly and die
<b>30–100 <math>\mu\text{m}</math> (most common)</b>	[H <sub>2</sub> O] < 20% (Orthodox)  Fabaceae, Solanaceae, Liliaceae	More than 1 hour	· Orthodox grains of this size resist thermal stress better during presentation and dispersal than smaller orthodox pollen · The time for rehydration and germination is also higher compared to smaller orthodox pollen
<b>100–200 <math>\mu\text{m}</math></b>	[H <sub>2</sub> O] < 20% (Recalcitrant)  Pumpkin, maize	A few minutes	· This category resists thermal and low relative humidity stresses better because of their larger size

<sup>(1)</sup>: Time depends on the status of pollen at stigma adhesion.

**Table 6.** Table showing the main categories of monad pollen, their size, shape at dispersal, time for rehydration and germination of orthodox (oval) and recalcitrant (spherical) pollen grains, including representative examples and some ecological consequences. The average pollen diameter is 30–100 micrometers with low water content. Orthodox and recalcitrant grains have ecological devices to reduce water loss during presentation and dispersal, e.g. pollen presentation by anthers that are enclosed by the flower corolla and exposing anthers outside the flower as for poricidal anthers.

metabolic activity of tapetum cells is required during meiosis for production of callose, a temporary cell wall that separates the microspores from the tetrad, and for biosynthesis and secretion of sporopollenin for the exine pollen cell wall [91–93]. Mutations that affect callose deposition and dissolution affect microspore development and fertility [94, 95]. The main tapetum nutritional activity occurs during the microspore stage and the first signs of



degeneration do not occur at the same stage in different species [96] but degeneration normally reaches completion near the end of the uni-nucleate microspore stage [97].

The secretory tapetum is the most common type [97]. The tapetum cells form the inner lining of the loculus and remain in place until they degenerate. In some plant species (e.g. Poaceae), the young microspores are found to attach themselves to the tapetum inner wall [35, 98]. In the secretory tapetum, the inner cell wall directed towards the loculus and the radial walls dissolve using a natural protoplasting event to facilitate the secretory function. Orbicules or Ubisch bodies are secreted towards the loculus by the tapetum cytoplasm; their function is not yet elucidated and only unproven hypotheses as to their role have been put forward [99–101]. During development, microspores are dispersed in the locular fluid, the volume of which can vary widely according to anther morphology (aseptate or septate) and the type of pollen dispersal units: more locular fluid is generally present in aseptate anthers and/or when pollen are dispersed as single units, while less fluid is present when pollen are dispersed as aggregates (Table 2) [82]. When released from the tetrad, pollen grains are in direct contact with the secretory tapetum [82]. The substances that are secreted in the locular fluid are neutral polysaccharides, pectins, proteins and lipids, and their relative proportion varies during pollen development [102]. The amount of locular fluid secreted depends also on the number, size and shape of the pollen grains and the dispersal unit (monads *vs.* polyads; Table 2).

Another form of tapetum is the amoeboid or periplasmodial tapetum which is, for example, found in the Asteraceae family [82]. In this case, the tapetum cell layer undergoes a reorganisation rather than degeneration during its early development. During meiosis, the tapetal cells form long extensions that engulf individual pollen mother cells. At the tetrad stage, the tapetum reorganises to form a periplasmodium which separates the individual young microspores and encloses them within a vacuole in the tapetal cytoplasm [103]. The amoeboid tapetum, better than the more common secretory type, illustrates the nurturing function of the tapetum.

The tapetum forms the interface between the sporophyte and the male gametophyte and is therefore in a strategic position to control reproductive development. Some of the substances entering the tapetum come from the external cell layers of the anther and other parts of the mother plant [104]. The mother plant supplies nutrients via the vascular bundle of the anther filament [84, 105]. Downloading occurs in the anther connective tissue cells and transport to the middle layer occurs symplastically [83]. The outer anther wall cells are connected via plasmodesmata, but the tapetum layer is symplastically isolated from other anther wall cells. Delivery of sugars into the tapetum requires apoplastic transport [55, 106, 107]. The apoplastic cell wall invertase gene is expressed in the tapetum and is responsible for mobilising sucrose into the tapetum cells [108, 109]. Repression of tapetal cell wall invertase activity and gene expression by different abiotic stresses blocks sugar transport to the pollen grains [108–112]. At least in some species nutritive substances are stored temporarily in the tapetum and are then absorbed by the developing pollen grains [102, 113].

The meiotic stage of pollen development is very sensitive to cold, heat and drought stress (Table 3) [25, 28, 109, 110, 114, 115]. It is likely that abiotic stresses at the time when the tapetum is metabolically most active interfere with the synthesis of pollen cell wall components and



the secretion of the locular fluid. This may cause abortion of the young microspores. The formation of the locular fluid is associated with an increase in pollen volume and increased vacuolisation, a process that is affected by water stress (Table 3) [28]. The presence of abundant locular fluid (e.g. Solanaceae and Poaceae) or its extreme reduction (e.g. some orchids, Fabaceae and Myrtaceae; Table 2) has so far not been correlated with higher or lower tolerance to drought stress. Plant species with a periplasmodial tapetum have a reduced volume of locular fluid. In this case, each microspore is engulfed in the tapetum cytoplasm, so pollen nutrition is direct and does not require an abundant locular fluid [82]. Abiotic stresses may interfere with tapetal PCD and affect its functionality [87]. Both premature and retarded degeneration of the tapetum cause pollen sterility [83, 87, 116–119]. Production of reactive oxygen species (ROS) has recently been implicated in the regulation of PCD timing in the tapetum [120]. ROS are produced in response to many abiotic stresses [121]. Premature tapetum degeneration is a major cause of pollen sterility and yield loss under abiotic stress conditions [118, 122–125]. Carbohydrate mobilisation to the tapetum and its genetic control may play an important role in guaranteeing pollen development under stress conditions. Anther sink strength is reduced in stress-sensitive species [108–110, 126]. At the same time, sugars appear to be redirected to other tissues, e.g. leading to starch accumulation in the endothecium layer of the anther wall [106, 107, 127]. The tapetum is a sporophytic tissue and its function is controlled by signals from the sporophyte (sugars, hormones). Improvement of stress tolerance in crop species will therefore require a better understanding of the effect of stress on the sporophyte, as well as on sporophyte–gametophyte communication.

<i>Stages</i>	<i>Stress type</i>	<i>Targeted stage and/or compartment</i>	<i>Defence mechanisms</i>
<b>Pollen meiosis &amp; further development</b>	· Water stress	· Tapetum, locular fluid formation	· Locular volume reduction
	· Water stress	· Microspore or bicellular pollen vacuolisation	· Anthers protected inside thick flower whorls
	· Low temperatures >0°C	· Cytoplasm activity and cyclosis	· Programmed developmental arrest
	· Heat stress >30°C	· Cytoplasm activity and cyclosis	· Programmed developmental arrest
<b>Anther desiccation</b>	· Rain	· Locular fluid fails to evaporate, anthers fail to dehisce	· Anthers are protected inside the corolla where pollen is exposed
	· Heat stress	· Carbohydrate metabolism	· Synthesis of heat shock proteins
	· Low temperatures	· Carbohydrate metabolism, cyclosis	· Presence of high amounts of LMW carbohydrate reserves
<b>Pollen presentation</b>	· Heat stress	· Pollen water content	· Pollen is presented inside the corolla
	· High/low relative humidity	· Pollen water content	· Pollen is presented inside the corolla and for a short time lapse
<b>Pollen dispersal</b>	· Heat stress	· Number of viable, dispersed grains	· Social plants with shorter pollen flight

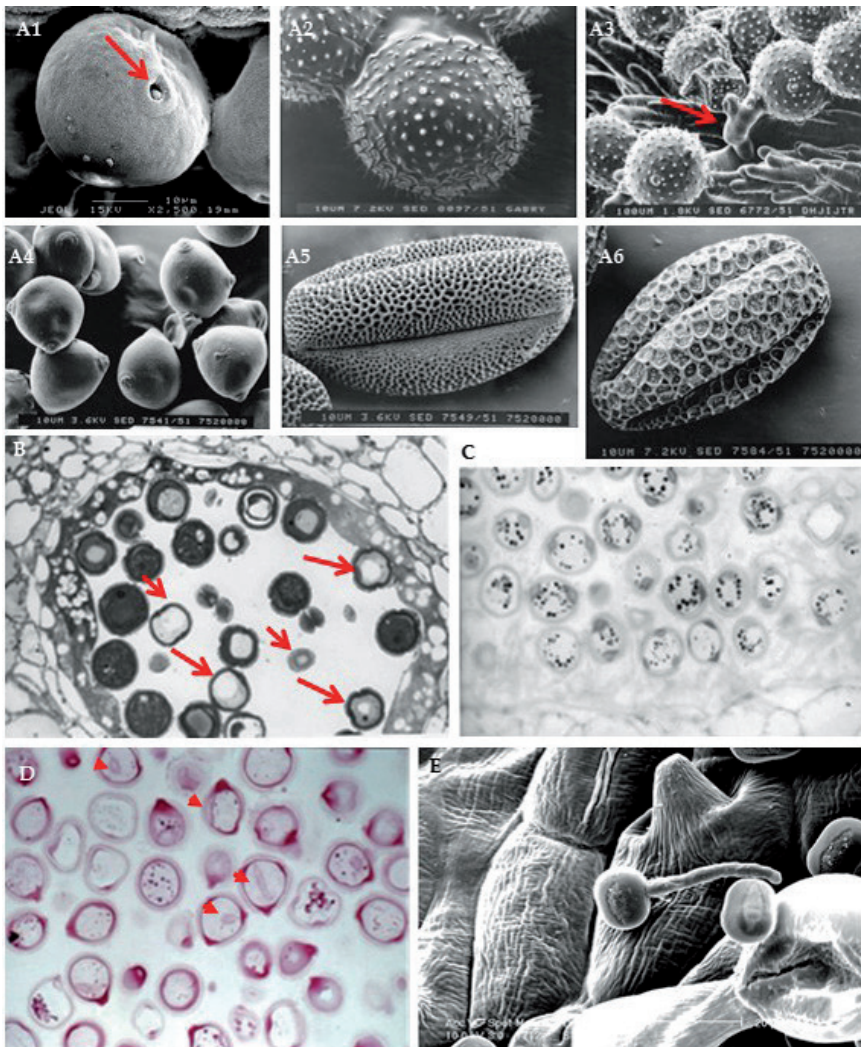
<i>Stages</i>	<i>Stress type</i>	<i>Targeted stage and/or compartment</i>	<i>Defence mechanisms</i>
	· High/low relative humidity	· Number of viable, dispersed grains	· Anthers can delay pollen dispersal

**Table 3.** Effect of abiotic stresses on different stages of pollen development, and possible evolutionary defence mechanisms of the male gametophyte.

5. The pollen cell wall

5.1. Exine

The synthesis of the pollen cell wall starts during meiosis and depends on the activity of the tapetum. The composition of the pollen wall is unique compared to other plant cell walls and shows species-specific diversity. The biodiversity in pollen cell walls is functionally important for the plant to distinguish its own pollen from that of other plants [128–131]. The pollen cell wall can vary physically and chemically to match environmental aspects of pollination. Pollen wall diversity serves a taxonomical value, forming the basis of palynology [132]. The extremely resistant and elastic outer exine wall has evolved to protect pollen during dispersal. Exine is deposited first to provide pollen grains with their distinctive and characteristic features (Figure 3A). Pollen cell wall organisation starts just before meiosis when meiocytes become surrounded by callose secreted by the tapetum [92, 93, 133]. The callose special cell wall (SCW) is formed during prophase and interphase and closes the cytotoxic channels that synchronise the first meiotic division. Exine is patterned under the callose layer and the microspore plasma membrane (primexine or exine precursor) at the end of meiosis (late tetrad stage) and is completed after the release of the microspores from the SCW at the end of the tetrad stage. The tapetum then produces callase, a  $\beta$ -1,3-D-glucanase enzyme responsible for dissolution of the callose wall, as well as sporopollenin precursors, a complex polymer of fatty acids and phenolic compounds. These are released in the loculus and polymerise on the primexine of the microspore following its release from the tetrad [132, 134, 135]. Mutant screens for impaired pollen walls in Arabidopsis revealed several genes involved in sporopollenin biosynthesis and most of these mutants are male sterile [92, 136–140]. Sporopollenin precursors are deposited by ABC transporters that are expressed in the tapetum at the early vacuolated microspore stage [134, 141]. Sporopollenin biosynthetic enzymes form a complex (‘metabolon’) in the endoplasmatic reticulum of the tapetum [142]. Recent ultrastructural studies reveal the involvement of specialised tapetum organelles, elaioplasts or tapetosomes, in exine wall deposition [140, 143]. Exine deposition is reduced, interrupted and can even be absent altogether in aquatic plants or plants living and pollinating in extremely wet environments [144]. The absence of exine in species having underwater pollination (e.g., seagrasses) is correlated with the fact that in water pollen grains do not undergo desiccation and have to remain hydrophilic; there is no developmental arrest and changes in shape and volume do not occur [145].



**Figure 3.** Different stages of pollen development in angiosperms.

A1-4: Scanning electron micrographs of mature desiccation-sensitive recalcitrant grains which are devoid of furrows.

A1: A rice pollen grain close to anthesis, showing the cell wall surface and the germination pore (arrow). A2: three pollen grains of *Lavatera arborea* (Malvaceae) kept together by pollen kitt, a viscous fluid covering the pores of the grains. A3-6: Pollen grains of different members of

the Cucurbitaceae family with recalcitrant pollen grains. A3: *Cucurbita pepo*, with a germinating pollen grain (arrow) taken 10' after pollination. A4: *Cucumis sativus*, a species with recalcitrant pollen grains. A5 and A6: *Bryonia dioica* and *Cytrullus lanatus* orthodox pollen grains with furrows.

B-E: Asynchrony in vacuolisation and starch storage in olive (*Olea europaea*) pollen grains. B: A section of anther at mid-bi-cellular stage during the second vacuolisation, with degenerating tapetum (toluidine blue O staining). The asynchrony of development of pollen is evident: grains have vacuoles of different sizes and some grains are degenerating (arrows). C: A section of an anther at mid-microspore stage at the first starch engorgement (stained with Periodic Acid Schiff). Starch grains have different sizes because of the asynchrony of starch storage. D: Section of an anther at the early bi-cellular stage and second starch engorgement (PAS stained). Grains have an asynchronous development with respect to starch engorgement and in some grains the generative cell (arrow heads) can be discerned because of the thin polysaccharide wall. E: Pollen grains of *Cerinthe major* (Boraginaceae) displaced by flower visitors on the corolla (SEM). Only one has emitted a pollen tube because of precocious rehydration due to high humidity during the night – probably indicating asynchronic development of the grains.

## 5.2. Intine

The exine wall is completed by the mid-microspore stage before the internal intine layer is deposited. Intine is less elastic and consists of a pecto-cellulose mixture. Intine synthesis also starts before the first mitotic division and is always completed by the time the vegetative and generative cells are formed [132, 146]. Mutagenesis approaches have identified genes involved in the biosynthesis of pectins for the intine cell wall [147–152]. Some of these genes are expressed in the tapetum and ABC transporters transfer intine components to the pollen grains [153]. Mutations affect pollen shape and fertility, as well as growth of the pollen tube. Pectin is the main component of intine and is secreted by the tapetum into the locular fluid. Accumulation is highest at the vacuolated microspore stage [146]. The *Brassica campestris* *Male Fertility2* and 9 (*BcMF2*, 9) genes encode novel polygalacturonase enzymes that play a role in pectin metabolism, intine formation and tapetum degradation [151, 152]. At pollen germination, the intine wall forms a continuum with the the pollen tube pectocellulose wall.

## 5.3. The role of the cell wall in regulating pollen size and shape

The pollen wall controls homeostasis of the cytoplasm and reduces fluctuations in pollen volume due to variations in water content. This is important during dispersal, when pollen is exposed to air. The characteristic exine wall furrows and surface pattern are crucial for the harmomegathic functions that regulate pollen shape during dehydration [154] (Figure 3 A5–6). After landing on the stigma, the pollen wall controls the rehydration process with water coming from the stigma in angiosperms or from the ovule in gymnosperms (pollination drop) [155].

The exine layer has generally one or more pores through which the pollen tube is emitted (Figure 3 A1, 3). When pollen pores are absent, the pollen tube is emitted at the site where the

pollen grain contacts the stigma surface. The pattern and distribution of the apertures are determined by the tetrad shape and callose deposition at the intersporal walls [156, 157]. The *Arabidopsis tam* mutant (*tardy asynchronous meiosis*) shows an altered cytoplasmic partitioning (cytokinesis) during tetrad formation and altered aperture patterning, suggesting that the last contact points between the cytoplasm of the future microspores during cytokinesis are the place where apertures are formed [158]. The number of pores per pollen grain can vary within one species and germination speed is positively correlated with pore number [159] and pollen water content at dispersal [160]. The intine wall is a continuous layer but is generally thicker and more elaborate at the pores and/or furrows to support the harmomegathic process [161]. Exine and intine have a similar thickness but in some cases intine, especially in the poral region, is much thicker and very pectin-rich, which may help in keeping pollen cytoplasm hydrated during dispersal [162–164].

#### 5.4. Pollenkitt

In some plant species, the surface of the pollen wall contains various amounts of pollenkitt, a viscous hydrophobic substance. The sticky nature of pollenkitt is thought to play a role in pollen adhesion to pollinators during dispersal [165, 166], but several other functions have been suggested [167]. Plants with zoophilous or entomophilous pollination, some of which having secondary anemophilous pollination, have exine cavities or ornamenations containing pollenkitt [168]. A simple and effective method was developed to reveal its presence or absence [169, 170]. The synthesis of pollenkitt is linked to tapetal degeneration [171] and plastids are implicated in its formation [104, 167]. In anemophilous plants, the plastids develop into elaioplasts which are resorbed by other tapetum cell components during degeneration. In entomophilous plants, the elaioplasts or tapetosomes (plastids accumulating lipids) are the more abundant organelles in the degenerating tapetum cytoplasm [167]. Tapetosomes are oil and flavonoid containing organelles in the tapetum that contribute to pollenkitt formation [172–174]. Pollenkitt is formed by the fusion of elaioplasts and spherosomes of tapetal cells during the late microspore stage [167]. After release in the locule, pollenkitt is deposited on the exine surface of the pollen grains, covering the exine ornamentations at the onset of anther dehydration [171]. In the entomophilous Brassicaceae family, elaioplasts are involved in forming tryphine, which plays a role in adhesion of pollen to the stigma [104] (Table 1). A conditionally male sterile mutant that affects tryphine production in *Arabidopsis* is affected in pollen-stigma recognition [69]. Pollenkitt consists mainly of saturated and unsaturated lipids, carotenoids, flavonoids, low molecular weight proteins and carbohydrates [167, 175]. An additional role of pollenkitt in biotic pollination could be in preventing water loss and other damage [167].

## 6. Pollen metabolism and development: role of vacuoles and plastids

### 6.1. Role of vacuoles

Vacuoles appearing at several stages of pollen development are correlated with metabolic activity. Pollen mother cells, like undifferentiated meristematic cells, are originally devoid of vacuoles but at telophase II small roundish vacuoles start to develop. Vacuolisation can occur once or twice (depending on species) during further stages of development [18]. Cyclic vacuolisation is always followed by storage of starch in amyloplasts (Figure 3 B–D), which then leads to disappearance of vacuoles and formation of new cytoplasm. Vacuolisation plays a role in increasing the volume of the pollen grain with the formation of new cytoplasmic components such as mitochondria, amyloplasts, other cell components and cytoplasmic reserves. Vacuolisation therefore reflects metabolic activity in the developing microspores. Vacuolisation is also associated with the storage of pectins during intine cell wall synthesis [146]. In *Arabidopsis* a large vacuole is formed by fusion of smaller vacuoles; this large vacuole is converted to smaller vacuoles again after the first mitotic division [176]. Lytic vacuoles (lysosomes) are formed to degrade mitochondria, ribosomes and plastids [18]. Mature pollen has only small vesicles filled with carbohydrates, but in species producing pollinia rather than single pollen (e.g. massulate orchids) small vacuoles with watery content are present. Reduced vacuolisation at maturity may be required to reduce pollen size during presentation and dispersal [33]. Pollen vacuolisation is also affected by abiotic stresses such as drought and temperature stresses (Table 3). Heat stress was shown to reduce pollen release from anthers [177]. Vacuoles also store metabolites such as sugars and play a role in regulating sugar homeostasis, metabolic activity and growth processes [178]. Sucrose cleavage into hexoses by vacuolar invertases can regulate osmotic potential of cells [179] and this can be used as a defence mechanism against stresses such as drought (Table 3). Abiotic stresses in *Arabidopsis* induce vacuolar invertase, as well as a tonoplast-associated monosaccharide transporter (*ESL1*) in vascular parenchyma cells [180]. Regulation of cellular sugar fluxes between cytoplasm and vacuoles is important to regulate osmotic potential and pollen hydration and this could play a role under environmental stress conditions. Vacuolar invertases that are expressed in pollen grains have been identified [108, 109], but their role in regulating pollen metabolism under stress conditions requires further investigation.

### 6.2. Role of plastids

Plastids are commonly present as undifferentiated pro-plastids at the end of meiosis. They divide later to differentiate and accumulate starch [181, 182]. Plastid division occurs in the vegetative cell of pollen before starch engorgement. Usually, there are one or two waves of starch accumulation in amyloplasts during pollen grain development in gymnosperms and angiosperms [162, 181]. In some plant species, pro-plastids in the generative cells are degraded by lysosomes immediately after the first haploid mitosis [183]. Plastids also store fatty acids and alcohol intermediates for pollen wall synthesis, as evidenced by the male sterile mutant *defective pollen wall (dpw)* [184]. Starch stored in the amyloplasts of the vegetative cell is in most plants hydrolysed before anther opening and pollen dispersal (Figure 3C). Physico-chemical



properties of starch in plants with two cycles of starch synthesis vary between and within species [185]. Mature pollen can be starchy or starchless, depending on the presence or absence of starch grains in the vegetative cell amyloplasts (Table 1). This can be characteristic for plant families [185]. In some plants that flower throughout the year in the same environment pollen grains are always starchless (e.g., *Mercurialis annua*) [186]. Vice versa, in the case of *Parietaria judaica* which flowers from springtime to autumn, the proportion of starchy and starchless grains varies according to the season [187].

6.3. Adjustment of osmotic pressure and water balance in pollen

Like soluble sugars, starch stored in plastids can play a role in adjusting osmotic pressure, particularly during presentation and dispersal (Table 3). Stored carbohydrates in plastids or in the cytoplasm, soluble or insoluble, can be used to adjust turgor pressure and protect grains against desiccation [188]. Many genes are involved in starch biosynthesis throughout pollen development [189]. Drought and temperature stresses can severely affect starch accumulation, and absence of starch in mature pollen can be an indicator of pollen sterility [109, 110, 115, 190–192]. Endogenous starch is consumed during the first phases of pollen tube emission when pollen tube growth is at the expense of pollen reserves [193, 194]. After this autotrophous phase, pollen grains obtain carbohydrates and other substances from the stigma and style. Starch presence is not a direct indication of carbohydrate reserves present in pollen; hydrolysis of starch from amyloplasts increases soluble sugar levels in the cytoplasm and sugars are stored in the vacuole [188, 193]. Carbohydrates derived from starch hydrolysis in starch-less pollen grains alleviates the effect of heat and humidity stress during presentation and dispersal [193]. Starch in plants is normally phosphorylated. A tomato mutant lacking starch phosphorylation activity (*Legwd*) fails to degrade starch for pollen germination, resulting in sterile pollen [182]. Hydrolysis of starch supplies soluble osmotically active sugars which, together with amino acids such as proline, provide osmotic adjustment [195] (Tables 3 and 4). Regulation of turgor pressure is an essential aspect of pollen tube growth and elongation [196]. Osmo-regulation during the late maturation phase may function in the dehydration of pollen. Pollen dehydration is associated with the induction of proteins that play a role in drought response: dehydrins, aquaporins, heat shock and LEA proteins [197]. High levels of osmotin expression in mature tobacco pollen is another indicator of osmotic stress response [198]. Potassium ions [199, 200] and phospholipids can also regulate osmotic pressure and cell swelling in pollen [201]. Regulation of pollen osmotic potential and water content and the role carbohydrates play in this process are clearly important in pollen development. Abiotic stresses (cold, heat and drought) during meiosis affect sink strength of the tapetum [109, 110, 126, 191, 202], but the dynamics of carbohydrate metabolism at the gametophyte level remain poorly understood.

Stage of development	Metabolic activity	Physiological effect
Ripening*	· Hydrolysis of starch · Synthesis soluble carbohydrates, amino acids, peptides	· Molecules increase pollen turgor pressure

Stage of development	Metabolic activity	Physiological effect
Desiccation*	<ul style="list-style-type: none"><li>· Resorption of water by phloem of filament</li><li>· Water redistributed to other flower parts</li><li>· Evaporation through anther cuticle</li><li>· Synthesis of protective molecules, proteins (LEA, dehydrins)</li></ul>	<ul style="list-style-type: none"><li>· Desiccation leads to higher pollen osmotic pressure</li><li>· Size of pollen grains affects desiccation</li></ul>
Presentation and dispersal	<ul style="list-style-type: none"><li>· Pollen water content is affected by humidity, temperature, content in osmotic molecules and their biosynthetic enzymes, levels of protective molecules</li></ul>	<ul style="list-style-type: none"><li>· High relative humidity causes precocious rehydration and extra-stigmatic pollen tube emission (especially in recalcitrant pollen)</li></ul>
Rehydration and pollen tube emission	<ul style="list-style-type: none"><li>· Time for rehydration/pollen tube emission depends on water content, osmotic molecules, biosynthetic enzymes, stigma adhesion</li></ul>	<ul style="list-style-type: none"><li>· The physiological state of the stigmatic surface plays an important role in pollen rehydration</li></ul>

(\*): The ripening and desiccation stages can – at least in some species – be totally or partially overlapping.

**Table 4.** Synthesis of osmotically active components in pollen, and their effect at different stages of development. Orthodox and recalcitrant grains could have a similar physiological behaviour until the onset of desiccation, but the amount and quality of the osmotic molecules and the activity of their biosynthetic enzymes distinguish the two categories in the later stages of pollen development.

7. Consequences of synchrony and asynchrony in pollen development: Pollen competition

The synchrony of the first meiotic division is likely due to the presence of cytomictic channels that unite the cytoplasm of all the meiocytes present at meiosis within a loculus [203, 204]. These channels close during the meiotic inter-phase and synchrony can be lost from the second meiotic division onwards; the two nuclei within one meiocyte can divide independently, but a certain proportion (30–40% in *Lycopersicum peruvianum*) can still divide synchronously [205]. Nevertheless, the dissolution of the callose wall that keeps the tetrad cells together is synchronous and is controlled by callase, which is produced and released by the surrounding tapetal cells [95]. Meiotic asynchrony can cause the second haploid mitosis and other cellular processes (vacuolisation, starch hydrolysis storage in plastids, intine formation) to be asynchronous [206]. In orchids, the process of microspore development is synchronous because of the persistence of cytomictic channels throughout meiosis, uniting all the microspores of a loculus until pollen mitosis [33]. Pollen maturation is not a synchronous event from the first mitotic division onwards. Because a large amount of ovules needs pollinating in the ovary, the staggered pollen maturation in orchids may offer an advantage in that overcrowding and competition of germinating pollen on the stigma can be avoided [33].



At anthesis, the release of microspores is controlled by the sporophyte; all pollen grains from a loculus are dehydrated and released irrespective of their developmental stage. In addition, pollen desiccation at the end of pollen development affects all pollen grains of the anther at the same time. The mix of asynchronic and synchronic events during pollen development results in a mixture of pollen grains at slightly different stages of maturity; the difference in physiological stage means that different pollen grains may contain different amount of reserves when they are released together during anthesis. Asynchrony in pollen development is obvious from differences in starch engorgement, vacuolation and pollen size at different stages of development (Figure 3 B–D). Asynchrony can also explain why *in vitro* pollen germination tests show variable efficiency, particularly for some plant species and for plants grown under stressful circumstances. *In vitro* pollen germination issues may reflect the *in vivo* situation; the higher the asynchrony of microspore development, the higher is the percentage of unviable and immature pollen grains at maturity. Environmental stresses such as drought, frost, heat, high humidity (rain and mist) exacerbate the degree of developmental asynchrony [114, 207], causing a further reduction in viable pollen count. Application of heat stress is a common technique used for improving yield of haploid embryos during microspore embryogenesis [208, 209]. Through induction of asynchrony in pollen development, abiotic stresses can affect the production of viable pollen at the gametophytic level. Very little is known about this process and its molecular and physiological basis.

Asynchronous development is responsible for pollen competition. Competition between grains occurs at different stages: during development, after rehydration on the stigma and during pollen tube growth. Asynchronous development, combined with the fact that the haploid pollen grains have a different genetic composition due to recombination of the sporophyte genome during meiosis, leads to differences in ability to compete during pollen development and this presents a continuous selective force throughout male gametophyte development. The tapetum cells secrete nutritive substances synchronously, but the asynchronous pollen grains have a different capacity to use these substances for development, causing competition. Asynchrony in development and differences in genetic composition then lead to competition during rehydration and pollen tube growth and the speed of pollen rehydration depends on the orientation of the pore(s) with respect to the stigma surface. The competition to be the first to fertilise the ovule(s) is an important selective force in plant sexual reproduction and played an important role in both plant and animal evolution [210].

## 8. Duration and continuity of pollen development

Pollen development is normally a continuous process that is interrupted only by pollen presentation and dispersal. Pollen meiosis takes only a few hours, but the duration of pollen development after meiosis can vary widely and depends on the plant species. As a rule, annuals develop pollen faster than perennials and woody species: pollen development takes 8 days for the herbaceous perennial *Lycopersicum peruvianum* [211] and approximately 7 days for geophytic *Lilium* species [212], while 18 days are required for the grass *Phalaris tuberosa* [213]. However, in some plants the process can be interrupted at various stages before presentation and dispersal. In some woody plants from temperate environments, the process

can be paused once or twice at the microspore or bicellular stages. The ability of pollen development to be interrupted is an adaptation mechanism to protect pollen against extreme environmental conditions during summer or winter. Interruptions are more likely to occur in plants where pollen development takes longer, especially in temperate climates where unexpected harsh weather conditions can occur. Some gymnosperms (e.g. *Juniperus communis*) and woody perennial angiosperms (e.g. birch, elder and hazelnut) that disperse their pollen at the end of winter differentiate their flower buds in autumn when environmental conditions are favourable [214, 215]. Under severe winter conditions, flower development is arrested and resumed in early spring. In hazelnuts, this interruption occurs at the bi-cellular stage [215]. The developing pollen grains appear dormant and anther metabolism is repressed. The influx of substances from the mother plant and the activity of anther wall chloroplasts are also reduced, suggesting that developmental arrest may be regulated by the mother plant. In some species, developmental arrest occurs prior to pollen meiosis. In some Mediterranean plants, flower buds develop during late spring but stay dormant during the hot and dry summer and development resumes in autumn [216]. The dioecious bay laurel (*Laurus nobilis*) flower buds of both sexes develop in early autumn, they pause development in winter and flower ripening and pollination occurs during early springtime [163]. It is not known how this developmental arrest of pollen development is controlled at the molecular and physiological level, but it provides a powerful defence mechanism to protect pollen and maintain fertility under sub-optimal climatic conditions.

## 9. Pollen dehydration, presentation and anther dehiscence

### 9.1. Orthodox pollen and cross-pollination

In cross-pollinating plants, the flower opens at anthesis and the pollen is dispersed to reach other plants (chasmogamy). To survive dispersal in the environment, pollen needs to be in a dehydrated state with low metabolic activity (Figure 4) [217, 218]. This is the case for orthodox pollen which is dehydration-tolerant and is dispersed with low water content (<20%). Orthodox pollen can travel over larger distances without losing viability [160, 219]. Near anthesis, rapid extension of the anther filament seals the xylem, interrupting sap flow to the anther. The phloem redistributes the locular content to other plant parts [160, 220, 221]. The epidermis and endothecium layers of the anther wall dehydrate and pollen grain hydration levels reach an equilibrium with the environment [222]. Environmental parameters such as temperature and relative air humidity influence pollen water content [186] and osmotic adjustment is used to balance water content in function of environmental conditions (Table 4) [27]. Orthodox pollen also has low metabolic homeostasis to prevent cellular damage during dispersal [160]. The duration of developmental arrest and viability of pollen depends on environmental conditions at dispersal and the type of reserve substances present in the pollen (Table 4) [27, 188]. These defence mechanisms protecting pollen grains during presentation, dispersal and pollination vary depending on the degree and duration of dehydration during dispersal and depend on whether plants are anemo- or zoophilous pollinators (Table 5) [27, 160, 223]. Relative air humidity can adversely affect pollination efficiency because absorption of water from the

environment can lead to precocious pollen tube emission when the correct hydrated state is reached (Figure 3E) [160]. Entomophilous pollen is also affected by compounds that are secreted by the insect carrier (e.g. bees) [224–226]. Plants producing orthodox pollen are potentially out-crossing; both out-crossing and self-pollination can occur in these plant species, unless there is a self-incompatibility system in place to prevent self-pollination [227].

Type of defence	Defence mechanism	Stage affected	
		Presentation	Dispersal
Structural, species-specific	· Close proximity of small herbaceous (social) plants		X
	· Grains protected inside anther until dispersal:		
	◦ Pollinia of massulate orchids	X	
	◦ Gradual dispersal, e.g. poricidal anthers of Ericaceae, Solanaceae		
	· Anthers exposing and protecting pollen inside the corolla	X	X
Ecological	· Pollen is presented during short periods with more favourable conditions		
	◦ Night pollination in dry habitats, e.g. Cactaceae	X	
	◦ During dry and sunny periods of the day, e.g. Gymnosperms		
Cytological	· Synthesis of molecules that protect pollen under stress conditions: carbohydrates, proteins and enzymes	X	X
	· Intine is thick and stores water, regulating the water content of the cytoplasm	X	X

**Table 5.** Common types of modalities present in different angiosperms in order to reduce and/or avoid the harmful effects of the environment during pollen presentation and dispersal.

9.2. Recalcitrant pollen and self-pollination

In self-pollinating plants, pollen does not have to travel far to pollinate and therefore does not need to undergo severe dehydration at maturity. These plants produce recalcitrant pollen grains which are dispersed with high relative water content (30–70%); pollen remains metabolically active at dispersal and continues to develop to the point of germination (reduced developmental arrest). Recalcitrant pollen grains are dehydration-sensitive and are typically very short-lived and highly sensitive to variation in relative air humidity [160] (Figure 3 A1 and A2; Figure 4). However, cross-pollination with recalcitrant pollen is possible but is restricted to proximate flowers only [228]. Some plant species produce both chasmogamic and cleistogamic flowers, thereby increasing the chance of reproductive success [227]. In crop

species (e.g. wheat, barley, rice), cleistogamic breeding systems may have been selected during domestication to limit gene flow and preserve preferred gene combinations [229–233]. The absence of pollen presentation in cleistogamic self-pollinating plants is thought to be a protection against abiotic stresses such as drought and heat, as pollen number is considered less of a constraint for pollination in cleistogamic compared to chasmogamic species [227, 234]. Some crop species still have both cleistogamic and chasmogamic varieties [232, 235, 236]. Cleistogamic rice varieties were shown to be more tolerant to heat stress at flowering compared to non-cleistogamic lines [237]. However, recalcitrant pollen (e.g. maize) can lose water quickly, especially at low air humidity [238] and many cleistogamic crop species (e.g. cereals, legumes, Solanaceae) have well-documented pollen sterility problems. These problems occur when plants experience stress at the young microspore stage or anthesis [25, 115, 191, 239–241]. Sterility in these cases may be inflicted earlier in development and may not be due to interference with pollen presentation and dispersal [242, 243]. This may indicate that cleistogamy *per se* may help avoiding pollen dispersal, but it may not offer protection against abiotic stresses that occur at other periods of flowering. Genetic manipulations and hybrid breeding in crop species have sparked renewed interest in controlling the breeding system of some crop species [231, 244–246]. Some progress has been made in recent years to identify the genes associated with the cleistogamy trait and flower opening in rice, wheat and barley [246–249]. This research will lead to a better understanding of the genetic basis of cleistogamy and chasmogamy and the implications for abiotic stress tolerance in crop plants.

### 9.3. Pollen size, shape and anther dehiscence

The size of mature pollen grains at dispersal varies from less than 15 to 200  $\mu\text{m}$  in diameter, with an average size of 70–100  $\mu\text{m}$  in the desiccated state. The variation in pollen size has been related to the stigma size [250] and does not always correlate with water content (Table 1) [160]. Pollen grain volume increases progressively from the young microspore stage to maturity but is generally restricted by available locular space and the type of pollen dispersal unit in different species [168, 251]. The dehydration process in orthodox pollen leads to a change in shape and size of pollen grains and the harmomegathic properties of the cell wall play an important role in this process (Figure 4; Table 6) [154]. Recalcitrant pollen do not have furrows to facilitate mechanical folding of the cell wall in response to dehydration and pollen remain spherical (Figure 4; Table 6).

Pollen release from the anther requires thickening of the secondary wall of the endothelial layer (= mechanical layer) and dehydration of the epidermis [163, 252–254] (Figure 1). Dehiscence mutants in *Arabidopsis* affect secondary wall thickening and cause male sterility; these mutants were shown to affect transcription factor genes *MYB26*, *NST1* and *NST2* [255–257]. Secondary cell wall thickening can also control temporary re-closure of the anther during rainy or misty weather [258, 259]. Dehydration of the epidermis is associated with increased abscisic acid (ABA) levels [260] and induction of dehydrin-like proteins [261]. Aquaporins regulate the movement of water during anther opening [262, 263]. Cells of the inter-locular septum are ruptured as a result of PCD, causing the joining of both locules of one theca – see Figures in Keijzer CJ [171] and Bonner LJ and Dickinson HG [264]. The locule volume increases and

absorption of the locular fluid is accelerated [220, 265–268]. The locular content is re-distributed to other plant parts via the elongating anther filament [160, 221] and aquaporins may facilitate the movement of water through the anther wall membranes [262]. A cell death response in the stomium then causes the anther to open and pollen grains dehisce with the help of tension caused by secondary wall thickening [253]. Depending on the plant species, the stomium can rupture completely (from the top of the anther to the base), partially, or form pores for pollen dispersal [266, 267, 269]. Plant hormones regulating senescence and cell death such as auxin, jasmonic acid and ethylene play a role in anther opening and pollen dehiscence [252, 270–273]. The elongation of the anther filament in some plant species is required to expose the anthers from the flower to facilitate dispersal (Table 5) [274].

Pollination in plants requires favourable interactions between pollen morphological factors and environmental conditions (Tables 6 and 7) [275]. The size and shape of pollen grains, together with the events in the anther wall regulating dehiscence all collaborate to determine desiccation time, pollen viability and pollination success (Tables 6 and 7). Variation in relative air humidity, together with abiotic stresses that affect relative humidity (heat, drought, cold stress), cause problems with pollen presentation, anther opening, dehiscence [276, 277] and pollen tube growth [32]. Precocious germination while still in the anther [278, 279], or while waiting for a pollinator to disperse the pollen (Figure 3E) [280, 281], is due to inappropriate levels of humidity. Plants have evolved clever species-specific adaptation mechanisms such as dehiscence at particular times of the day [282], dispersal as single pollen or aggregates [168, 283], active dispersal by explosive forces rupturing the anther (e.g. *Ricinus communis*) and interaction with grooming insects [284, 285].

<i>Pollen stages</i>	<i>Processes affected by abiotic stress</i>
· Meiosis	· Drought prevents secretion of the locular fluid
· Tetrad stage	· Drought during pollen development influences volume increase of the different floral parts
<b>Pollen development</b>	· High/low temperatures and drought lead to consumption of starch reserves and carbohydrate starvation in anthers, affecting sugar delivery to pollen
· Microspore stage	
· First haploid mitosis (asynchronous)	
· Bi-cellular/tri-cellular stage	
<b>Anther and pollen desiccation</b>	· Drought during anther and pollen desiccation prevents transport of locular fluid water to other floral parts
	· High air relative humidity prevents anther and pollen desiccation
	· Too low relative humidity of the air accelerates anther and pollen desiccation
<b>Pollen presentation <sup>(*)</sup></b>	· Too low air relative humidity affects pollen viability, especially in recalcitrant species

Pollen stages	Processes affected by abiotic stress
	<ul style="list-style-type: none"> <li>· High air relative humidity induces precocious rehydration of pollen grains and pollen tube emission</li> <li>· Low or high temperature extends or reduces pollen presentation</li> <li>· Drought reduces flower longevity</li> </ul>
Pollen dispersal	<ul style="list-style-type: none"> <li>· Low air relative humidity affects pollen viability</li> <li>· High air relative humidity induces precocious pollen rehydration and can prevent anther dehiscence</li> <li>· Some volatile compounds emitted by bees affect pollen viability</li> </ul>
Pollen rehydration	<ul style="list-style-type: none"> <li>· Low air relative humidity prevents pollen rehydration and affects water availability from the stigma</li> </ul>

(\*): This phase is absent when pollen leaves the anther when it opens (e.g. Poaceae) or is launched from the anther (e.g. castor bean)

**Table 7.** Stages of male gametophyte development in angiosperms and processes affected by abiotic stresses.

**9.4. Breeding systems and pollen:ovule ratio**

The pollen:ovule ratio (P/O) has traditionally been used as a rough estimator of plant breeding systems (Cruden 2000), but little is known about the effect of environmental stresses on this ratio. When pollen is dispersed in aggregates of hundreds of grains (e.g. massulate orchids), the locular space is restricted and limited locular fluid limits nutrition and volume increase [34]. Changes in pollen volume can be measured under optimal or stressed conditions [28, 286]. Pollen dispersed as aggregates provides greater pollination success when the ovary contains multiple ovules [168, 287] and water loss during presentation and dispersal under heat and drought conditions affects only the externally exposed pollen grains and not the internal ones. To improve pollination success, some plants produce different types of pollen (different size, shape, colour, carbohydrate and water content) in one flower. One type, fecundative pollen, is fertile and able to emit the pollen tube and fertilise, while the other type is sterile nutritive pollen that serves as a reward for pollinators who – at the same time – get dusted with fecundative pollen [288]. The flower morphology can affect accessibility of pollen by different pollinators. Self-incompatible dimorphic *Primula* species have two different flower types with reciprocal anther and style length, producing pollen with different water content depending on the position and exposure of the anthers with respect to the corolla tube [289, 290]. Three flower types, producing three types of pollen grains, occur in trimorphic species (e.g. *Lythrum salicaria*) [289, 291]. The differences in flower morphology result in non-random mating patterns in plant populations and may play an important role in pollinator selection and adaptation to different environments [292, 293].

## 10. Conclusions

The diversity in adaptation mechanisms available in nature to secure reproductive success in angiosperms is considerable (Tables 6 and 7). This diversity can serve as a valuable resource to advance our insights into stress adaptation mechanisms that will benefit breeding strategies for crop species. Cytological and morphological studies, combined with other science disciplines (physiology, genetics and genomics) will continue to improve our understanding of pollen development and its adaptation to the environment. The number of genes and mutants involved in male reproduction is steadily increasing [294], but several research areas require further attention:

- Two crucial stages of anther development are strongly affected by environmental conditions. Until dehiscence, anthers are protected by the calyx and corolla, but for pollen dispersal, anthers need to be exposed. Both flower opening and anther dehiscence are strongly influenced by the environment [171, 184]. Secondly, the secretion functions of the anther tapetum are strongly affected by abiotic stresses. Tapetum cells are highly specialised secretion cells that lose their inner cell walls, effectively turning them into natural protoplasts and making them very vulnerable to water stress [82]. Drought stress at meiosis reduces locular fluid secretion [115], causing malnutrition and asynchrony of the developing pollen grains. Interestingly, some plant species are adapted to growth in very arid environments and expose pollen during the hot season, yet always have a very reduced volume of locular fluid (e.g. *Eucalyptus* and *Acacia* species in Australia). *Eucalyptus rhodantha* can resist temperatures higher than 50°C for several days without significant reduction in pollen viability [295]. It is important to understand how the tapetum of these plants manages to provide sufficient nutrients to sustain pollen development. The available locular space and the capacity to store locular fluid are abundant in plants dispersing solitary pollen, but very reduced when grains are dispersed as polyads (e.g. pollinia) [82]. Abundant locular fluid is considered a 'primitive' character in land plants and is a characteristic shared by all gymnosperms [160]. During evolution, locular volume has been gradually reduced and/or replaced by polyad dispersal, possibly as an adaptation to drier environments or to allow pollen presentation over longer periods of time (e.g. massulate orchids) [33, 105]. Orchid species can have monad or pollinia dispersal units [296]; the more primitive species have monad and tetrad pollen with abundant locular fluid, while the more evolved species disperse pollinia and produce very little locular fluid [33]. It remains to be established whether/how reduced locular fluid volume and compound pollen dispersed over longer periods of time could benefit sexual reproduction in arid environments and orchid species could be used for this research. Various other adaptation mechanisms could alleviate the effect of abiotic stresses, including shorter duration of pollen development, night – rather than day – pollination, deposition of a thicker protective intine wall, dispersal of compound rather than single pollen can all reduce the negative effect of stresses [160, 279].
- The control of pollen number, size and shape is another poorly understood aspect of pollen development. Pollen development is started (meiosis) and terminated (anther dehiscence) at a fixed moment. When environmental conditions induce various degrees of asynchrony

throughout pollen development, this leads to decreased numbers of viable pollen at anthesis. Larger pollen numbers could be obtained in plants with larger anthers. Anther size is a trait that has been used for selection of cold tolerance in rice [297] and the growth hormone gibberellic acid plays an important role in controlling stamen development [298]. Elucidating the mechanism of interrupting or pausing pollen development under unfavourable conditions may also provide useful information about avoiding stress damage. Understanding these mechanisms will require a better understanding of the signals driving gametophyte development *per se*. The haploid genome of the male gametophyte is derived from the sporophyte, but very little is known about its functionality in regulating pollen-specific development and metabolism. Achieving this challenge is now within reach, thanks to sensitive new-generation transcriptome analysis techniques [29, 71, 299].

- It is important to understand the signalling mechanisms between mother plant and male gametophyte. Some crucial steps in pollen development (meiosis, tapetal activity and anther dehiscence) are clearly under sporophytic control. The high sensitivity to abiotic stresses of the meiotic, young microspore and anthesis stages indicates that sporophytic signals are critical in controlling male gametophyte development. Stress-proofing crop plants may therefore have to start by understanding the sporophyte signals (sink-source relationships, carbohydrate and hormone signalling, control of PCD during tapetum degeneration and anther dehiscence). It has been known for some time that treatments with one stress or with the stress hormone abscisic acid (ABA) can improve tolerance to another stress – a process called stress ‘hardening’ or ‘priming’ [300–305]. More recent studies in rice have shown that stress treatments at the vegetative stage can affect abiotic stress tolerance during flowering and reactive oxygen species (ROS) signalling could play a role in this sporophytic signalling event [306]. But evidence for involvement of genomic imprinting and epigenetic mechanisms in sporophyte-gametophyte signalling is also mounting [307–309].
- The importance of air relative humidity in pollen development has so far been grossly underestimated. The growing area of staple crops such as cereals is increasingly extending into environments that require different adaptations of pollen development. For instance, tropical rice is grown in temperate climate zones and temperate climate wheat is grown in humid tropical environments [310–312]. Air humidity and climatic conditions modifying atmospheric humidity (rain, fog, cold, heat and drought) have a dramatic effect on plant species producing orthodox and recalcitrant pollen, causing asynchrony and reducing pollen number and fertility. The dynamics of water relations and osmotic regulation in pollen grains and their interactions with the environment are research topics that need urgent attention. Adapting the breeding system of crop species (self- versus cross-pollination) may offer opportunities for improved protection of pollen during dispersal, but the trade-offs between chasmogamy and cleistogamy in terms of abiotic stress tolerance require more detailed investigations.



## 11. Appendix

Term	Definition
• Meiocyte, pollen mother cell	Sporophytic cell in the centre of the anther that is destined to undergo meiosis and generate haploid pollen grains.
• Microspore	Alternative term used to refer to a pollen grain, but mainly used for the earlier uni-nucleate stages of pollen development. Young microspores refer to the first stage of pollen development, i.e. the cells released from the tetrad after meiosis. Microspores develop into the male gametophyte.
• Tapetum	Inner layer of the anther wall surrounding the meiocytes and loculus of the anther. Consists of secretory apoptotic cells that nourish and regulate pollen development. The tapetum degenerates, producing pollenkitt and other substances that cause pollen grains to aggregate.
• Cleistogamy/chasmogamy	Cleistogamy refers to automatic self-pollinating plants that do not open their flowers before pollen dispersal. In contrast, chasmogamy refers to plants that do open their flowers to release pollen in the environment for dispersal by animals or wind (potential cross-pollinators).
• Pollen Dispersal Unit	Pollen grains can be dispersed as single grains (monads) or as aggregates of several pollen grains kept together by viscous fluids or filaments (polyads). Tetrads derived from a single meiocyte can stay together in groups of four, united by common walls. In orchids, many packed tetrads can be arranged in different ways to form pollinia containing hundreds or thousands of pollen grains.
• Monads, polyads, pollinia	See pollen dispersal unit.
• Orthodox/recalcitrant pollen	Based on water content at dispersal, pollen grains can be classified as orthodox or recalcitrant. Orthodox pollen is desiccation-resistant and has a low water content (2–20%). Recalcitrant pollen is desiccation-sensitive, with water content between 20% and 50%. Orthodox and recalcitrant pollen grains both have advantages and disadvantages at pollination.
• Male germ unit	Is the association of a vegetative nucleus with a generative cell or two sperm cells to form a functional male reproductive unit in angiosperms. The term 'unit' reflects the close connection between the sperm cells and the vegetative nucleus.
• Septate/aseptate anthers	In septate anthers, in contrast to aseptate anthers, the meiocytes are separated by a wall (septum), dividing the locule in smaller compartments filled with pollen grains.
• Pollen presentation	Is the process of pollen exposure for dispersal to reach the stigma for pollination. Pollen presentation involves interaction between the anther and other floral parts. Primary presentation occurs when pollen grains are

Term	Definition
	exposed in the anther. Secondary presentation involves developmental relocation of pollen from the anther to another floral organ. Pollen grains are not presented by the anther when they are launched using different mechanisms.
• Zoophilous, entomophilous and anemophilous pollen	Pollen dispersal by animals, insects and wind, respectively.
• Pollen engorgement	Pollen maturation is associated with accumulation of starch granules in the cytoplasm. This process is called engorgement.
• Harmomegathy	The capacity of pollen grains to change shape in response to a decrease in volume during dehydration and prior to the development arrest state. This dynamic process is controlled by the mechanical properties of the cell wall (furrows) and can be reversed by rehydration on the stigma. When pores are absent, this increase and decrease in volume is due to the elasticity of exine and intine.
• Furrow	A fold region where the exine cell wall has reduced thickness, whilst intine is thicker. Furrows allow the cell wall to collapse to comply with the decrease in pollen volume during dehydration and increase volume during rehydration.
• Development arrest state	Term used to indicate the state of physiological and metabolic arrest when pollen grains reduce water content before dispersal.
• Of the locular fluid changes	Central cavity in the anther where pollen grains develop. The loculus is filled with the locular fluid which is secreted by the tapetum and serves to nurture pollen. In cross-section, anthers show four locules. The composition of the locule fluid changes during pollen development, and before anther dehiscence the fluid is reabsorbed by the filament or other floral parts to allow pollen presentation. The locular fluid is abundant in anthers with monad and tetrad pollen, but is reduced in species with pollinia or where grains are tightly packed.
• Mechanical layer	External cell layer of the anther wall where, after tapetum degeneration, cells develop lignified wall thickenings. The mechanical layer is responsible for anther opening and pollen exposure
• Pollenkitt	Hydrophobic glue derived from the degeneration of the tapetum, composed of saturated and unsaturated lipids, carotenoids, flavonoids, proteins and carbohydrates. Pollenkitt makes grains stick to the anther, to the pollinator body and to the stigma surface.
• Pollen viability	Term used to indicate the percentage of viable pollen (i.e., able to emit pollen tubes and fertilise). Pollen viability can be assessed by hand pollination, in vitro germination and several methods evaluating physico-

Term	Definition
	chemical parameters of pollen (e.g., plasma membrane intactness, the presence/abundance of some molecules or enzymes).
• Sporopollenin	Chemically and biologically resistant and elastic substance forming the building block of the exine cell wall. Sporopollenin consists of a mixture of carotene and carotenoid esters.
• Exine	External discontinuous cell wall of pollen grains. Exine is elastic, is composed of sporopollenin and has an opening called the pollen germination pore or aperture.
• Intine	Inner continuous pecto-cellulosic wall of pollen grains. The intine structure is more complex at the apertures and furrows where pollen tubes will be emitted. The intine wall becomes continuous with the pecto-cellulosic wall of the pollen tube during germination.
• Callose	Polymer of glucose residues linked together through $\beta$ -1,3-linkages. Callose is deposited during meiosis to separate the meiocytes and tetrad cells during meiosis. Callose represents a molecular filter to separate cells and is degraded by callase separated by the tapetum ( $\beta$ -1,3-glucanase).
• Pollen desiccation and water content	Pollen grains desiccate before dispersal to reach equilibrium with environmental conditions. Metabolism is slowed down to better resist the negative effects of the environment (high or low temperature and relative humidity). Orthodox and recalcitrant pollen have different water contents at dispersal.
• Secreted by the gymnosperm ovule	Liquid secreted by the ovule and exposed outside the stigma. When pollen grains land in the pollination drop, they rehydrate and germinate.
• Pollination syndrome	Term to describe the pollination traits that plants use in their natural environment to move from one flower to another, using different vectors. Plant can use abiotic (wind, water), as well as biotic (bees, birds) vectors to transfer pollen grains.
• Pollen competition	Haploid pollen grains differ in their genomic composition (recombination during meiosis) and therefore behave differently during development, pollen tube germination and in response to environmental challenges. This leads to competition between pollen grains. Pollen competition is an example of rapid Darwinian selection.

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